INVESTIGATING NOVEL APPROACHES TO NOROVIRUS DIAGNOSIS

A thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Medicine

by

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To

My family: Mum and Dad, Stuart, Matthew, and Stephen. This is the product of your hard work more than mine.

Acknowledgements

This study was conducted with the assistance of many professional colleagues, not all of who it is possible to personally acknowledged here.

The project followed on from a study carried out by the late Professor Tony Hart. I would like to thank him for his support and enthusiasm.

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Declaration

This thesis is the result of my own work. The material presented here has not been presented and is not being presented, either wholly or in part for any other degree or qualification. Some aspects of the thesis were carried out in collaboration with other people and reference has been made to these people in the main body of the thesis. In particular:

- The initial idea to study the oral detection of norovirus was given to me by the late Professor Hart. I believe the idea came to him via colleagues studying Feline Calicivirus at Liverpool University, School of Veterinary Science.
- The real time RT-PCR of samples collected for the study of the oral diagnosis of norovirus was carried out by staff in the Royal Liverpool University Hospital Virology Laboratory, principally Lynne Ashton.
- The collection of samples for the evaluation of new immunological norovirus tests were provided by Professor Ricardo Gurgel and his staff in Aracaju.
- The real time RT-PCR of the Brazilian samples was carried out by Winifred Dove and Angela Booth.

Abbreviations

AMAU	Acute Medical Assessment Unit
AP	Arrow Park Hospital
BG	Broadgreen Hospital
bp	base pairs
cDNA	complementary DNA
^{0}C	degrees Celsius
C.I.	confidence interval
CT	Liverpool Heart and Chest Hospital
CV	coefficient of variation
Da	Dalton
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
EM	electron microscopy
GI	genogroup I
GII	genogroup II
HPLC	high performance liquid chromatography
ICG	immunochromatographic
Ig	immunoglobulin
km	kilometres
l	litre
M	molar
MAbs	monoclonal antibodies
mg	milligram

ml	millilitre
NJ	neighbour joining
nt	nucleotide
O.D.	optical density
ORF	open reading frame
p	probability
PCR	polymerase chain reaction
ppm	parts per million
NPV	negative predictive value
PPV	positive predictive value
RH	Royal Liverpool University Hospital
RNA	ribonucleic acid
ROC	receiver operating characteristics
rpm	revolutions per minute
RT	reverse transciptase/transcription
S.D.	standard deviation
STARD	standards for reporting of diagnostic accuracy
TBE	tris base, boric acid and EDTA
UK	United Kingdom
μl	microlitre
UV	ultraviolet
WH	Whiston Hospital

ABSTRACT

To control outbreaks of norovirus infection and to understand better the importance of norovirus in the developing world, appropriate diagnostic tests must be used. In an attempt to improve norovirus diagnostics, two separate investigations were undertaken. The first investigated the use of oral samples for the diagnosis of norovirus infection in adult hospitalised patients. The second investigated two recently developed immunological tests, the RIDASCREEN ELISA and RIDAQUICK immunochromatographic (ICG) test, for their detection of norovirus.

The first study was conducted at five hospitals in Merseyside, UK, in the winter of 2008/9. In total, 66 paired oral and faecal samples were collected from patients with symptoms consistent with norovirus infection. Oral samples were mouthwashes of sterile water. Of the 66 mouthwashes, 59 (89%) had norovirus confirmed with the reference standard, RT-PCR of faeces. Of these, 14 (24%: 95% C.I. 14-37%) had norovirus detected in mouthwashes by one step real time RT-PCR. The detection of norovirus in mouthwashes was associated with the presence of vomiting ($p = 0.1$). This study has demonstrated for the first time the detection of norovirus in mouthwash samples. This test is not currently sensitive enough for routine application in clinical diagnostics.

The second study was undertaken on faecal samples from children with diarrhoea in Brazil. The study compared new immunological norovirus tests, the RIDASCREEN ELISA and RIDAQUICK ICG test, against an established norovirus ELISA (IDEIA ELISA). The evaluation was carried out on 96 positive and 116 negative samples for norovirus by PCR. This showed the RIDASCREEN ELISA (R-Biopharm, Darmstadt, Germany) was more sensitive (64%) than the IDEIA ELISA (Oxoid, Ely, UK), (49%), with RT-PCR as the reference standard ($p = <0.01$). The sensitivity of the RIDASCREEN ELISA could be increased to 90% in hospitalised children by reducing the specificity of the test to 80%. The RIDASCREEN ELISA is therefore a reasonable means of testing samples for norovirus, prior to molecular analysis, in molecular epidemiological surveys of children hospitalised with gastroenteritis. The RIDASCREEN ELISA and RIDAQUICK ICG were 78% and 88% sensitive respectively in detecting the GII.4 strain of norovirus. The GII.4 strain is responsible for most outbreaks of infection in hospitals. Both the RISASCREEN and RIDAQUICK tests were 100% specific; therefore, multiple samples can be tested in an outbreak to identify the presence of norovirus, leading to an increase in the tests sensitivity without compromising test specificity. The RIDASCREEN and RIDAQUICK tests are therefore likely to be useful in diagnosing nosocomial outbreaks of norovirus infection.

In addition to the diagnostic evaluations, the molecular epidemiology of noroviruses collected in both studies (Liverpool, UK and Aracaju, Brazil) was investigated. In the Merseyside hospitals, individual hospital trusts had distinct strains of GII.4 norovirus, despite all 8 distinct strains detected being very similar. The Brazilian analysis demonstrated a genetically stable GII.4 strain predominated. This strain was detected across a wide geographic area throughout the study period (over 1 year). This requires further investigation to define if this represents person to person transmission or semi-permanent environmental sources of infection.

Continued improvements in the diagnosis of norovirus will allow an improved understanding of the global burden and epidemiology of norovirus gastroenteritis. This may lead to improved control strategies being developed for norovirus infection reducing the costs and morbidity associated with norovirus infection.

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CHAPTER 1

Introduction and objectives

1.1 Viral gastroenteritis

Gastroenteritis is a ubiquitous clinical condition associated with substantial morbidity and mortality, particularly in children living in developing countries. An estimation of paediatric mortality concluded that of ten million deaths in children under five years of age globally between 2000 and 2003, 18 % were related to gastrointestinal infection (Bryce et al. 2005). While bacteria and parasites are significant causes of gastroenteritis, viruses are the major cause (Kapikian 1993). Many viruses are found in the gastrointestinal tract, some are confirmed agents of gastroenteritis, whereas the role of other viruses is less clear. In addition to norovirus; rotavirus, astrovirus, adenovirus and sapovirus are the principal known viral aetiological agents of gastroenteritis. These are presented below in brief, having been presented in detailed reviews elsewhere (Matson and Szucs 2003; Wilhelmi et al. 2003; Clark and McKendrick 2004).

Rotavirus has a genome consisting of segmental double stranded (ds) RNA. The virion is 70nm in diameter and nonenveloped with transmission via the faecal-oral route. Rotavirus is the principal viral cause of paediatric diarrhoea and mainly causes illness in children aged six months to two years. The incubation period is 1-2 days with fever and vomiting preceding the onset of diarrhoea. Diarrhoea lasts for 1-4 days and significant volume depletion can occur. Diagnosis can be made by electron microscopy (EM), latex agglutination tests, enzyme linked immunosorbant assay (ELISA) and polymerase chain reaction (PCR) testing. Treatment is symptomatic including fluid replacement.

Astrovirus has a genome consisting of a single stranded RNA. The virion is 28-35nm in diameter and nonenveloped with transmission by the faecal oral route. Astrovirus causes infection mainly in children with an incubation period of 2-4 days. The infection results in an illness which is a mild version of rotavirus. Diagnosis can be made by EM, ELISA and PCR. Treatment is symptomatic including fluid replacement.

Enteric adenovirus has a genome consisting of a dsDNA. The virion is 90–100nm in diameter and nonenveloped. Enteric adenoviruses mainly affect children and the incubation period is 8-10 days. Fever and vomiting occur in addition to diarrhoea which lasts 3-11 days. Diagnosis is by EM, ELISA and PCR. Treatment is symptomatic including fluid replacement.

Sapovirus has a single stranded RNA virus genome. The virion is 41-46nm in diameter and is nonenveloped. Sapovirus is a cause of acute gastroenteritis which is self limiting. Infection with sapovirus is most commonly reported in institutional outbreaks affecting children. Diagnosis is by EM, ELISA and PCR. Treatment is symptomatic including fluid replacement.

Viral gastroenteritis causes most morbidity and mortality in paediatric populations. Rotavirus is the principal agent of paediatric gastroenteritis in developed and developing countries with an estimated 444000 deaths per year (Parashar et al. 2006). Norovirus is the second most common cause of paediatric gastroenteritis and is also an important cause of outbreaks of gastroenteritis in all ages.

1.1.1 Norovirus introduction

Norovirus is a cause of gastroenteritis. It commonly causes outbreaks of infection because it has a high secondary attack rate (Lopman et al. 2004). Norovirus gastroenteritis is normally of short duration, approximately 48 hours, and has limited morbidity or mortality in healthy adults. Its discovery in 1972 required the use of immune electron microscopy (Kapikian 2000) as it cannot be grown in cell culture (Duizer et al. 2004). Since then it has been shown to be one of the principal agents of infectious gastroenteritis worldwide (de Wit et al. 2001; Koopmans 2008). Today the medical implications of norovirus lie in two main areas. Firstly, in the developed world it causes large institutional outbreaks with significant morbidity and cost implications (Lopman et al. 2004). Secondly, it is a cause of gastroenteritis in the developing world where it contributes to the burden of intestinal disease in children.

1.1.2 Norovirus genome

Noroviruses consist of small round virions of 27 to 35nm in diameter and possess a single-stranded, positive sense, polyadenylated RNA genome of 7,400 to 7,700 nucleotides. The genome is divided into three open reading frames (ORFs) (Figure 1.1).

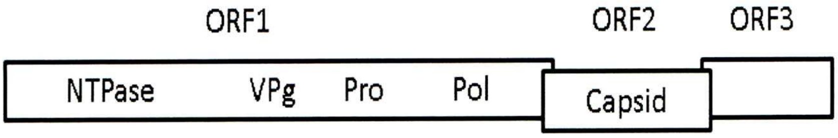


Figure 1.1: Division of the norovirus genome into three open reading frames (ORFs).

ORF1 encodes a large polyprotein which undergoes proteolytic cleavage to produce viral proteins that are homologous to proteins from other single-stranded RNA viruses. These include a nucleoside triphosphatase, a helicase enzyme which ensures uncoiling of dsRNA, a 3C-like protease and an RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein with an apparent molecular mass of 58kDa. The capsid protein has several domains, including the N domain facing the interior of the capsid, the S domain involving the formation of the icosahedral shell and the P domain forming the prominent protrusion emanating from the shell. A component of the P domain, the P2 domain, forms the antigenic part of the virion. ORF3 encodes a small protein abundant in basic amino acids. Although the precise role of the ORF3 protein is unknown, it is likely that it is a minor structural protein that interacts with the genome RNA when virion formation occurs (Green et al. 2001)

The norovirus genome, as an RNA genome, undergoes frequent genetic changes when replication occurs. This allows rapid viral evolution which has been reported at rates of 4.3×10^{-3} nucleotide substitutions/site/year, similar to other RNA viruses (Bok et al. 2009). This rapid rate of evolution allows antigenic change which has been suggested as a mechanism of escaping from population immunity. This antigenic change has been compared to antigenic drift seen in influenza virus. A component of influenza viruses' antigenic drift is positive selection. Positively selected sites are those that have higher rates of nonsynonymous than synonymous changes in protein coding sequences relative to other regions of the genome. Positive selection indicates immune driven mechanisms of genetic evolution are present. (Shih et al. 2007). Work by Bok *et al* has investigated norovirus genomes for the presence of positive selection. This has been found in the capsid protein (ORF2), mainly in the shell domain but at one site within the protruding P2 domain (Bok et al. 2009). Further study of ongoing norovirus genetic changes are needed to better understand the process of norovirus evolution.

Investigation of the variation between norovirus genomes has also taken place. This has been with the aim of determining where norovirus RNA is similar and divergent between strains. This has shown that norovirus isolates are most similar at the junction of ORF1/2 and most dissimilar at the P2 region. This had led to the ORF1/2 junction being targeted for the production of broadly reactive norovirus PCR primers (Katayama et al. 2002).

1.1.3 Classification

The family *Caliciviridae* is composed of four genera of virus, *Norovirus, Sapovirus, Vesivirus, and Lagovirus.* The *Norovirus* genus is split into genogroups based on characterization of the capsid gene. Five genogroups have been described with genogroups I and II, and rarely IV, being associated with human infection. These genogroups have been classified into over 30 genotypes by RNA sequence analysis of the capsid sequence (Figure 1.2). Genogroup and genotype classifications have more recently been made by structural alignments of the capsid amino acid sequences (Table 1.1) (Zheng et al. 2006; Koopmans 2008). This alignment method demonstrated that genogroups have pairwise difference ranges from 40-62%.

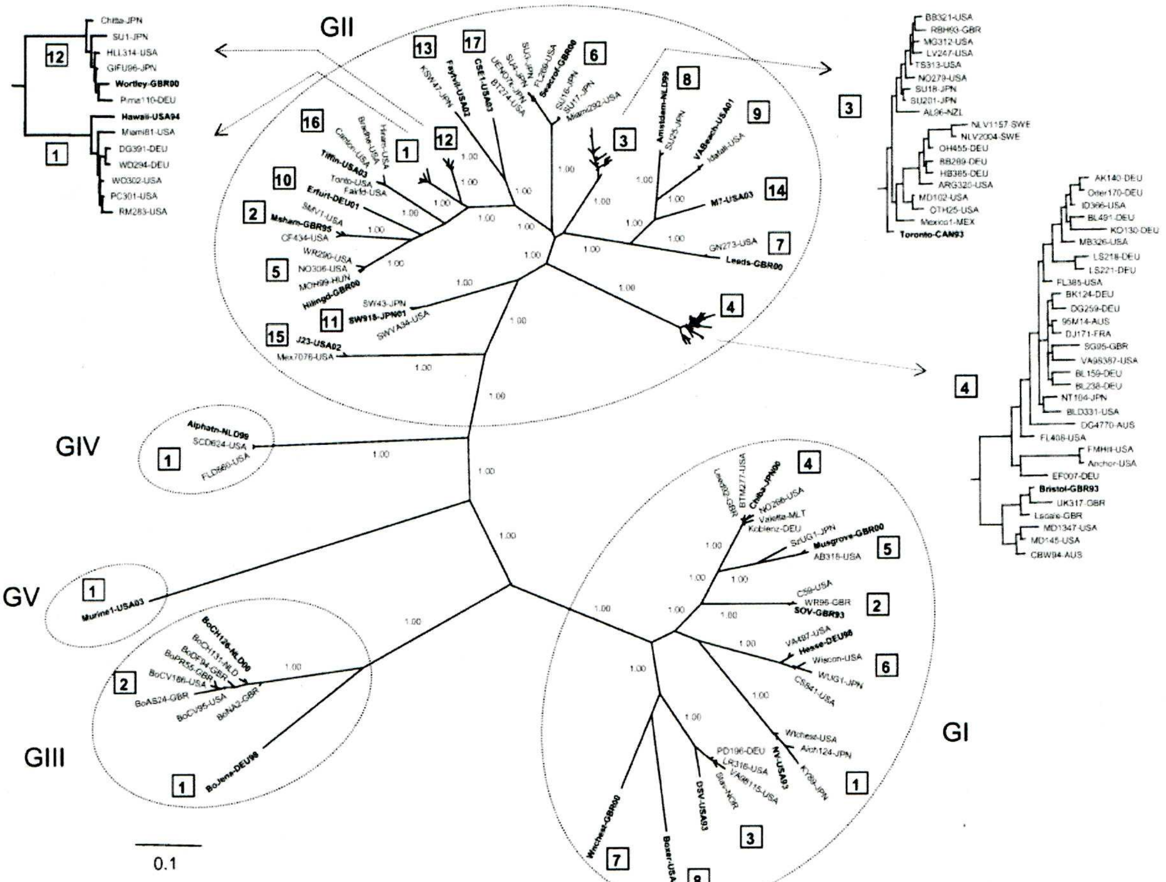


Figure 1.2: Norovirus genogroup and genotype classification produced by genomic sequence analysis of the capsid region. Figure reproduced from Zheng *et a*l. (Zheng et al. 2006). Measurement bar represents nucleotide substitutions per base.

Table 1.1: Pairwise difference ranges between Norovirus genogroups (%).

	G1	G2	G3	G4	G5
G1		48.54–55.21	45.62–52.29	48.75–53.75	55.62–58.33
G2	50.20–57.06		50.21–54.79	41.88–48.33	55.21–58.54
G3	47.57–53.94	52.07–56.89		52.50–54.38	56.25–58.12
G4	52.11–56.11	44.91–51.61	54.88–56.58		56.04
G5	58.14–60.74	58.17–61.41	57.74–59.68	58.56–58.75	

Differences were calculated by using an uncorrected distance method with a 141 capsid amino acid sequence alignment (top right portion) and with a 164 capsid amino acid sequence alignment (bottom left portion). Table reproduced from Zheng *et al* (Zheng et al. 2006).

1.1.4 Clinical and molecular epidemiology

Clinical epidemiology: Norovirus is global in its distribution, infecting people of all ages. It predominantly causes infection in the community: In a UK based prospective study 1562 community cases were predicted to occur for every one reported to the national surveillance scheme. It was also found that 6.3 cases of norovirus attended a general practitioner with acute gastroenteritis for every person reported to the national surveillance scheme. A rate of 12.5 community cases per 1000 person years was demonstrated in this study, making norovirus the most common cause of gastroenteritis identified (Wheeler et al. 1999). These may have been underestimates as electron microscopy was used to detect norovirus which is recognised as an insensitive test for the detection of norovirus. A Dutch study prospectively investigated community gastroenteritis, also finding norovirus to be the most common cause of gastroenteritis. It identified that in patients with gastroenteritis in the community, 16.1% (10.9% adjusted for control group) of cases were infected with norovirus. This study analysed norovirus infection in different age groups and showed norovirus was detected in patients with gastroenteritis at prevalence's of 14-18% in <18 year olds and ≥65 year olds, but 7% in 18-64 year olds (de Wit et al. 2001).

Norovirus, given its mild morbidity, commonly presents to the medical profession when an outbreak occurs and large numbers of people are affected. A review of the location of these outbreaks reported to the Center for Disease Control (CDC) in the USA showed 39% in restaurants; 29% in nursing home/hospitals; 12% schools/day care centres; 10% in holiday settings, including cruise ships; and 9% in other settings (Parashar et al. 2001). In the UK infection is mainly seen (80%) in health care institutions (Lopman et al. 2003). These numbers are likely to differ due characteristics of a countries health care facilities e.g. hospital isolation facilities. The impact of outbreaks in the developed world is greatest when medical institutions are affected as patients are already unwell and there are significant associated cost implications (Lopman et al. 2004).

Clinical studies have shown that norovirus infection is most common in the first two years of life, and principally in the first year of life (de Wit et al. 2001). Seroepidemiological studies have shown that infection occurs worldwide, from a young age, and is more prevalent in developing vs. developed countries with antibodies lowest in the first two years of life rising thereafter (Cubitt et al. 1987; Cubitt et al. 1998; Nakata et al. 1998; O'Ryan et al. 1998; Peasey et al. 2004). The low seroprevalence in the first two years of life may reflect that this age group lack immunity to norovirus which may explain why they have a higher rate of infection compared to other age groups. It is also possible that serological assays are more able to detect norovirus antibodies which developed in response to older strains of norovirus. Older children may have antibodies to older strains and younger children may have antibodies to more recent strains of norovirus which immunological assays are less able to detect.

In the developed world a number of reports have investigated the rates of detection of norovirus in the faeces. A wide range of infection rates are detected, as would be expected with a virus with a high secondary attack rate leading to outbreaks of infection. Reported rates range from 6-40% in children attending hospital with gastroenteritis with a mean rate of 15% (Koopmans 2008). It is difficult to differentiate asymptomatic shedding from infection in these studies as asymptomatic shedding rates can be significant. Shedding rates have varied from 1% to 30% (O'Ryan et al. 2000; Castilho et al. 2006) but are more commonly seen at around 8-10% (Farkas et al. 2000; Monica et al. 2007; Reither et al. 2007).

A seasonal pattern is often detected in norovirus epidemiological studies with norovirus infection most commonly detected in winter. This is reported in studies from the USA, Europe, Asia and Australasia (Mounts et al. 2000; Iritani et al. 2003; Dey et al. 2007; Siebenga et al. 2009). This winter pattern is not exclusive, summer time peaks of infection have also been detected (Lopman et al. 2003; Chhabra et al. 2009). In some studies no seasonal peak of norovirus infection is detected (O'Ryan et al. 2000; Kirkwood et al. 2005). Further work is needed to understand what drives the observed seasonality of norovirus infections.

Molecular epidemiology: Currently there are global epidemics of norovirus GII.4 strains which show genetic drift analogous to that seen in influenza A viruses, first reported by Siebenga *et al* (Siebenga et al. 2007) (Figure 1.3). At any one time highly homogeneous virus is detected globally suggesting global spread. This was first noted in 1999 (Noel et al. 1999). Since that time a definitive answer has not emerged to explain the possible mechanism of this global spread. Speculated mechanisms, in addition to person to person spread, include food

(Widdowson et al. 2005) and water contamination (Lodder and de Roda Husman 2005; Maunula et al. 2005).

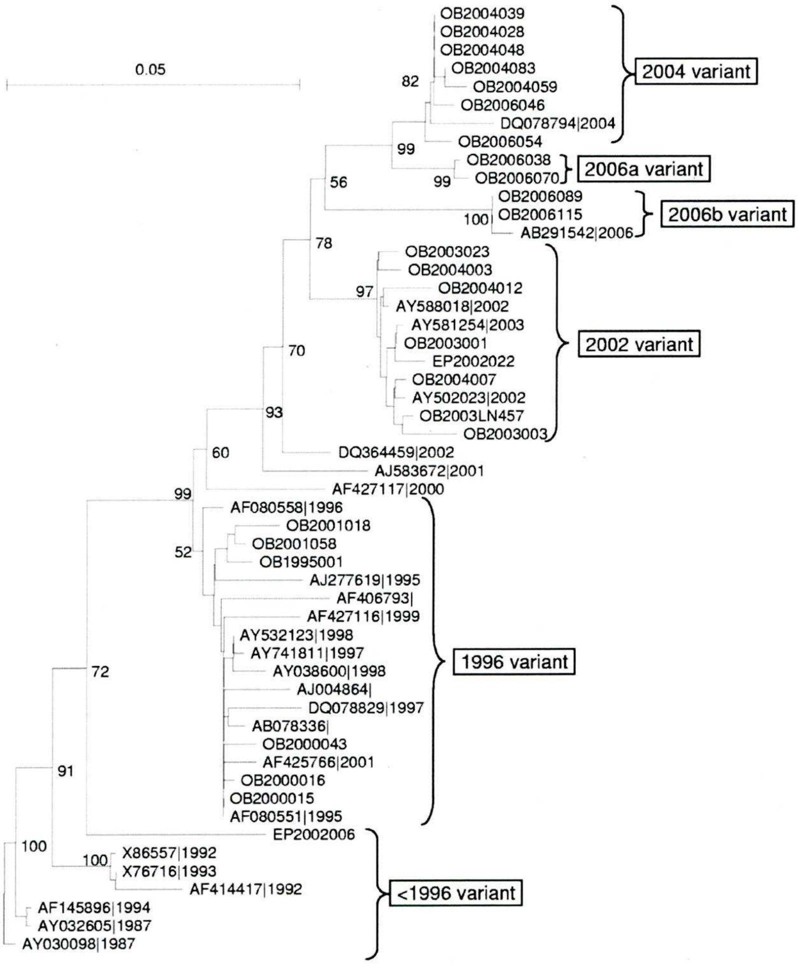


Figure 1.3: Neighbour-joining tree for capsid amino acid sequences of norovirus identified from 1996-2006 (Netherlands). Figure reproduced from Siebenga *et al* (Siebenga et al. 2007).

1.1.5 Clinical features

The clinical features associated with norovirus infection can differ in specific cohorts, principally: children, adults in the community, elderly adults in institutions and immunosuppressed patients.

Paediatric norovirus infection: An acute onset of vomiting is followed by diarrhoea of short duration (up to 48 hours). In children the illness is essentially not different from healthy adults and consists of diarrhoea, vomiting, nausea, abdominal cramps, fever, and malaise. Vomiting is though more common in children/young adults compared to elderly adults. Infection with norovirus can be asymptomatic. One small study showed 11/14 children infected with norovirus during a norovirus outbreak were asymptomatic (Matson et al. 1990). Asymptomatic shedding of norovirus in children has also been reported with rates of 6-30% (Farkas et al. 2000; Akihara et al. 2005; Garcia et al. 2006; Amar et al. 2007). These studies all used PCR to detect norovirus which is a highly sensitive method of detecting norovirus. Studies which used electron microscopy to detect norovirus had previously underestimated the amount of asymptomatic norovirus shedding that occurs (Amar et al. 2007). Community outbreaks have been reported where a point source has acted as an uncontrolled challenge to allow documentation of the effect of norovirus infection. Symptoms in a community outbreak associated with a water fountain in Holland were: abdominal pain 89%, vomiting 70%, nausea 65%, headache 70% and diarrhoea 60%. A mean duration of illness of 2 days was reported (Hoebe et al. 2004). The clinical features of children attending hospital with norovirus detectable in faeces have been well reported. In a study of paediatric diarrhoea in Vietnam symptoms/signs were: diarrhoea 100%, vomiting 66% and fever of $\geq 38.5^0$C in 33% with a mean duration of diarrhoea and vomiting: 4.4 and 1.5 days respectively (Nguyen et al.

2008). In a study of paediatric gastroenteritis in Nicaragua symptoms/signs were: diarrhoea 46%, vomiting 54%, dehydration 43% and fever of $\geq 38^{0}$C in 29% (Bucardo et al. 2008) (Bucardo et al. 2008; Nguyen et al. 2008). The rates of diarrhoea reported in these studies vary because inclusion criteria varied. Mortality from norovirus, excluding occasional case reports (Thiele et al. 2005), is likely to be due to its contribution to disease burden in children with malnutrition or other illness. Attributable mortality rates are unknown but have the potential to be significant in the developing world.

Adult norovirus infection in the community: An acute onset of vomiting and diarrhoea of up to 48 hours duration, but not requiring medical intervention, is typical. In a study of community based norovirus infection in Spain symptoms/signs were: vomiting 64%, diarrhoea 85% and fever 40% (Arias et al.). Community outbreaks of norovirus are commonly caused by foodborne transmission of norovirus. Symptoms in foodborne outbreaks have been reported in a Swedish study where symptoms/signs were: vomiting 64%, diarrhoea 71% and fever in 44% (Gotz et al. 2001). Foodborne outbreaks have also been reported in India where symptoms/signs were: vomiting 64%, diarrhoea 92% and fever in 43% and in Italy where symptoms/signs were: vomiting 85%, diarrhoea 58% and fever in 16% (Girish et al. 2002; Prato et al. 2004).

Adult norovirus infection in institutions: Symptomology is published for elderly nursing home residents, an example of symptoms are diarrhoea (73%), vomiting (40%), abdominal pain (33%), malaise (30%) and fever (10%) (Grmek Kosnik et al. 2007). Lopman *et al* compared elderly (75-84 years old) nursing home residents with elderly hospital residents. It was found hospitalised residents had a longer duration of diarrhoea, median duration 3 days in hospitalised patients compared to 2 days in nursing home residents (Figure 1.4) (Lopman et al. 2004).

Comparing the clinical features between infection in institutions and in the community by foodborne outbreaks suggest vomiting is more common. It is difficult though to compare these figures as the age of those affected differs and case detection will vary in the two different settings.

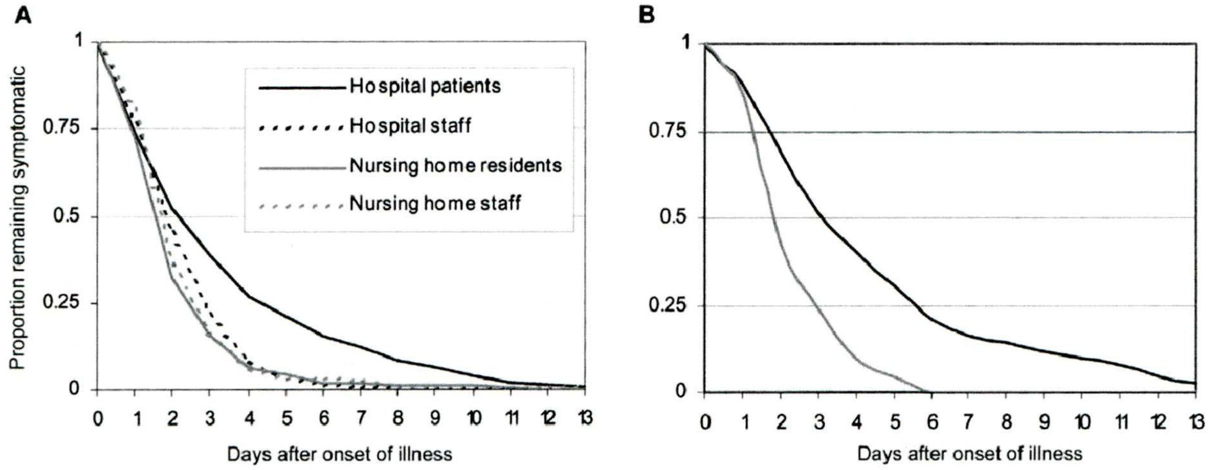


Figure 1.4: Duration of symptoms after norovirus infection. A, all cases associated with outbreaks of norovirus infection; B, microbiologically confirmed. Figure reproduced from Lopman *et al* (Lopman et al. 2004).

The mortality from norovirus infection has been estimated to be approximately 70 deaths per year in the UK in those ≥65 years old (Harris et al. 2008).

The complications of norovirus infection in hospitalised patients was assessed by Mattner *et al* who showed dehydration confirmed by >10% increase in creatinine in 22/84 of patients as well as 18/84 having detectable potassium loss of >10% (Mattner et al. 2006).

Immunosuppressed patients: The numbers of immunosuppressed patients is increasing with the spread of HIV infection and increased use of immunosuppressive agents associated with transplants and chemotherapy treatments. These patients are at risk of infection in the same way as the rest of the population but it is suggested they can develop persistent gastroenteritis of many months duration (Gallimore et al. 2004; Lee et al. 2008; Ludwig et al. 2008).

1.1.6 Clinical management

Norovirus, being an acute gastroenteritis with minimal mortality in healthy adults, does not routinely require specific therapy. In those patients severely affected, the young and the elderly in particular, supportive therapy (fluid and electrolyte replacement, temperature control and analgesia) may need to be more intensive. The need for these supportive measures was shown by an assessment of hospitalised patients which showed serum potassium falls of 10% occurred in 21% of those affected and creatinine rises of 10% occurred in 26% of patients. Risk factors for the development of potassium falls were cardiovascular disease and renal transplantation. Immunosuppression was a risk factor for creatinine increases of >10%. In view of the common occurrence of vomiting the management of these complications may require intravenous infusions to replace fluids and electrolytes (Mattner et al. 2006). In patients who are on immunosuppressive therapy a reduction in the immunosuppression should be considered. The use of antivirals or immunoglobulin has not been proven to have any effect in the patients though immunoglobulin is occasionally used where diarrhoea has been prolonged (Florescu et al. 2008).

1.1.7 Laboratory diagnosis

Norovirus was initially detected by immune electron microscopy (EM) when the virus was first identified in 1972. This offered an opportunity to diagnose rapidly from faecal samples (Kapikian 1993). A characteristic appearance of the 27nm virus by EM is shown in Figure 1.5. Its surface shows multiple cup shaped depressions, these cups (Latin=calix) have given the *Caliciviridae* family its name. EM only detects virus when there are 10^6 particles per ml of stool (Dolin et al. 1972). This gives it a sensitivity of approximately 30% and restricts diagnosis to the acute stage of infection.

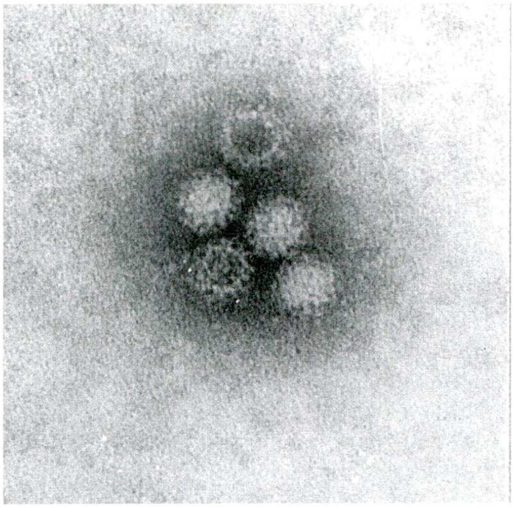


Figure 1.5: Norovirus particles by electron microscopy. Picture provided by Büchen-Osmond, C. to the International Committee on Taxonomy of Viruses (http://www.ncbi.nlm.nih.gov/ ICTVdb/WIntkey/Images/em_norwa.gif)

Routine laboratory diagnosis of norovirus can also be undertaken by ELISA. This has a variable sensitivity and specificity when compared to PCR diagnosis but an approximate sensitivity of 60% is reported (Vipond et al. 2000; Dimitriadis et al. 2006; Gray et al. 2007).

An alternative to ELISA testing is immunochromatographic (ICG) testing. This allows small numbers of samples to be investigated quickly. Both ELISA and ICG testing have continued to be developed and new commercial products continue to be produced. Immunological methods of diagnosing norovirus infection are optimal in the first 72 hours of infection as viral load is reduced after this time (Graham et al. 1994; Okhuysen et al. 1995). Antigen detection methods are presented in detail in Chapter Three (3.1.1).

Norovirus PCR is now the standard method used for the clinical detection of norovirus and is described in detail in the literature (Atmar and Estes 2001; Vinje et al. 2003). It is highly sensitive and specific but does have some limitations in that the primers may not detect all strains of virus (Jiang et al. 1999). Two separate PCR primer sets are commonly used to detect norovirus genogroup I and II (Rolfe et al. 2007). Given norovirus is an RNA virus, and PCR requires DNA, the RNA must first be reverse transcribed into cDNA from the RNA. After reverse transcription (RT) the cDNA is replicated by PCR leading to the accumulation of PRC product which can be detected by gel electrophoresis (called qualitative or end point PCR). This method of detecting PCR product is being replaced by real time RT-PCR. Real time RT-PCR is more sensitive than qualitative PCR (Pang et al. 2004). Real time RT-PCR uses two methods for detecting PCR product. One is the dye, SYBR Green, which binds non-specifically to double stranded DNA (PCR product) and fluoresces. The other method is to use probes which bind specifically to PCR products. These probes fluoresce when DNA polymerase excises them following specific DNA binding. RT-PCR using probes is therefore highly specific and it has also been found to be more sensitive than using SYBR Green (Gunson and Carman 2005). The probe based method of norovirus PCR method has also been adapted so the RT and the PCR can be carried out in a single reaction tube, so called one step real time RT-PCR (Rolfe et al. 2007).

1.1.8 Modes of transmission.

Faecal oral transmission: Many foodborne outbreaks of norovirus are reported in the literature. These outbreaks have been caused by food handlers (de Wit et al. 2007) and by people eating food contaminated prior to preparation in a kitchen. This contamination is principally related to faecal contamination of food through the use of manure on crops (Showell et al. 2007) and through bivalve shellfishes (filter feeders) e.g. oysters which are taken from areas exposed to human sewage (Huppatz et al. 2008). Attack rates in bivalve shellfish exposures and food handler outbreaks are high. The high attack rates are more impressive when considering that 20% of the population are immune to infection and approximately 50% of those susceptible may have asymptomatic infection (Lindesmith et al. 2003): In 95 oyster-associated outbreaks 58% were symptomatically infected and in 195 food handler-associated outbreaks 47% were symptomatically infected (Noda et al. 2008).

Outbreaks of norovirus are mainly caused by genogroup II viruses. Recent work has suggested that GII virus is more prevalent than GI due to a higher viral load being excreted in the faeces (Chan et al. 2006); this higher viral load potentially increasing the number of transmission events the GII viruses cause.

Airborne transmission: Norovirus is present in vomitus, with an estimated concentration of 30,000,000 norovirus particles per 30ml of vomit. It is a 27nm particle, a size that remains airborne indefinitely (Morawska 2006). Given that the infectious dose of norovirus is 10-100 particles, it seems feasible that norovirus could be transmitted by the airborne route. Unfortunately, reports of airborne transmission in the literature have been unable to exclude the role of fomite transmission (Sawyer et al. 1988; Marks et al. 2000).

Tracking transmission of virus: Transmission of outbreaks can be tracked through sequence analysis of virus in affected patients. Accumulation of mutations is seen in virus transmitted from person to person compared to a single source outbreak where limited virus mutation is detected (Dingle 2004).

1.1.9 Prevention and control

Preventing norovirus infection is difficult because vomiting and diarrhoea spread the virus in the environment and a low infectious dose is required to cause infection. The best way to prevent infection is therefore to prevent exposure. This can be achieved by preventing symptomatic people from coming into contact with susceptible people. An example is stopping admission of patients with gastroenteritis to hospitals. Once exposure has occurred standard precautions are recommended e.g. hand washing. The virus is unculturable so it is not known what disinfection procedures remove the viruses' ability to infect cells/people. A number of similar viruses e.g. feline calicivirus, have been investigated as surrogates (Duizer et al. 2004; Cannon et al. 2006; Bae and Schwab 2008). These suggest alcohol is ineffective and high concentrations of bleach, >100ppm, are needed. Alternative disinfectants that may be effective include Quaternary Ammonium Compounds and Hydrogen peroxide (Barker et al. 2004; Duizer et al. 2004; Tree et al. 2005; Shin and Sobsey 2008). Given it is difficult to prevent infection in those exposed to norovirus it is important to cohort these patients for 48 hours after exposure as they may be incubating infection during this time. Guidance is available from national bodies on how best to control norovirus outbreaks e.g. Health Protection Agency, UK (Chadwick et al. 2000). The only control measure documented to play a significant role in limiting spread of noroviruses in hospitals is ward closure (Lopman et al. 2004).

1.1.10 Pathogenesis

The pathogenesis of norovirus infection has undergone limited investigation due to the difficulty in accessing the site of infection, the small bowel and pylorus of the stomach. In the 1970s, two norovirus challenge studies did obtain gastrointestinal tissue via endoscopy. Reported histological changes in duodenal-jejunal junction biopsies showed reduced crypt-to-villous ratio, abnormal villous absorptive cells, increased cellularity of lamina propria and polymorphonuclear leucocytes in the lamina propria (Schreiber et al. 1974). The second study showed an intact mucosa, moderately blunted villi, and a moderate inflammatory cell infiltrate in the lamina propria consisting of polymorphonuclear and mononuclear cells with appearances having returned to normal at 2-3 weeks (Dolin et al. 1975). A number of animal models are reported but the relevance of these to human infection is unclear (Cheetham et al. 2006; Ward et al. 2006; Mumphrey et al. 2007; Souza et al. 2008).

1.1.11 Susceptibility and immune responses to norovirus

In humans the immune response has been investigated by completing challenge studies in healthy adults. These studies have allowed collection of clinical samples (faeces, saliva and serum) to investigate immune responses. Also, clinical outcomes of challenges, including repeat challenges, have been assessed. Challenge studies of healthy adults have shown that the clinical response to norovirus challenge is variable. Clinical responses include no clinical illness to a range of mild to severe gastroenteritis. No clinical illness can be due to the absence of a cell receptor in the gastrointestinal tract that the virus attaches to prior to causing cell infection. This receptor is known as H antigen (H-type-1). Its formation is part of the ABO blood group system which is expressed differently in the gastrointestinal tract to the blood. The biosynthetic pathway is shown in detail in Figure 1.6. Non secretors do not have an enzyme for the production of H-type-1, being homozygote recessive for the FUT2 gene responsible for the production of α(1,2) fucosyltransferase which creates H-type-1. *In vitro* studies have shown norovirus binds to H-type-1 supporting the hypothesis this is the gastrointestinal receptor for norovirus. Non secretors are therefore called this in view of their lack of secreted H antigen onto the gastrointestinal epithelium (Lindesmith et al. 2003). Approximately 20% of Caucasians are non secretors of H antigen.

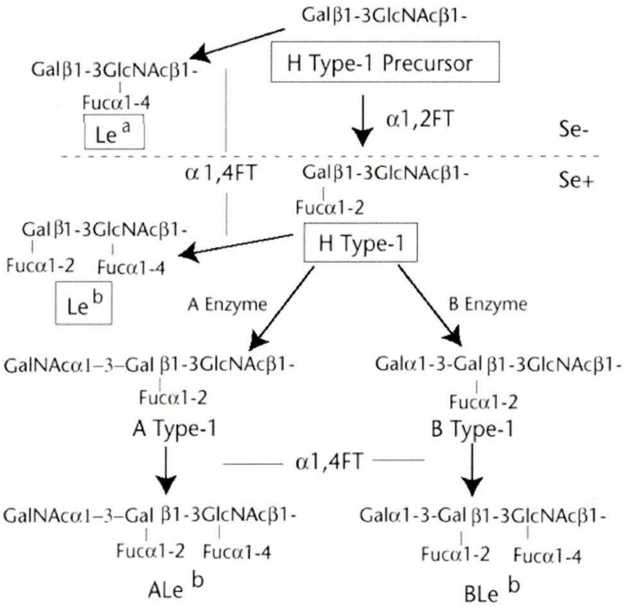


Figure 1.6: Biosynthetic pathway for H antigen required by norovirus to cause human infection, reproduced from Lindesmith *et al* (Lindesmith et al. 2003).

Subjects who are secretors of H antigen may also not become clinically unwell when challenged with norovirus, as well as not becoming infected (infection defined as detection of norovirus in faeces). This group of subjects have an early elevated level of salivary IgA, compared to secretors who become infected with norovirus (Figure 1.7).

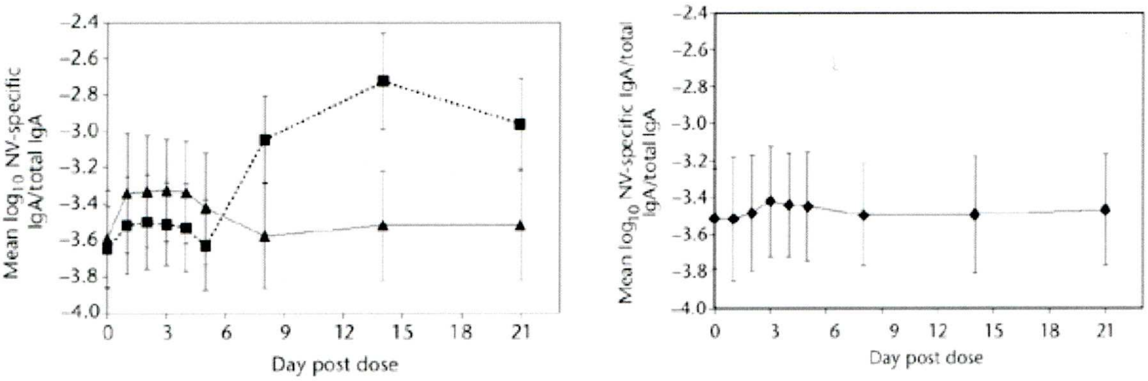


Figure 1.7: Salivary IgA responses in patients challenged with norovirus on day 0. ▲ – susceptible patients who were infected, ■ - susceptible patients who were not infected ◆ unsusceptible patients. Reproduced from Lindesmith *et al* (Lindesmith et al. 2003).

In those subjects who become infected with norovirus, confirmed by detection of norovirus in faeces, only half will become symptomatic. These findings were reported by Lindesmith *et al* and are summarised in Figure 1.8 (Lindesmith et al. 2003).

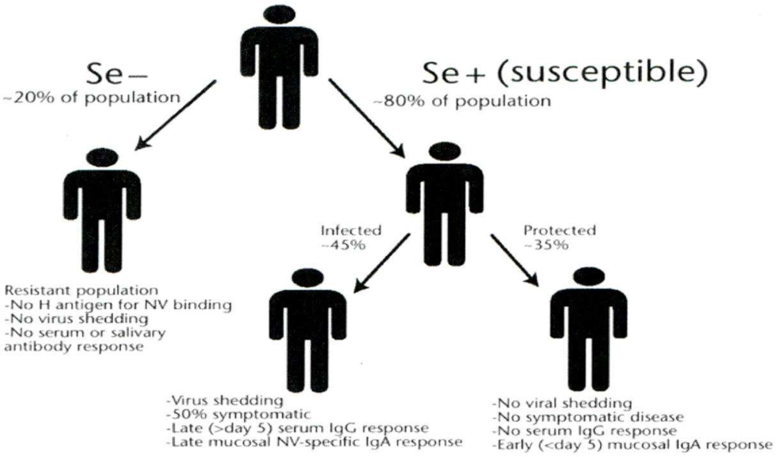


Figure 1.8: Clinical and immunological outcomes in norovirus challenge studies of healthy adults. Figure reproduced from Lindesmith *et al* (Lindesmith et al. 2003).

Immunity to norovirus has been investigated by a limited number of small studies. These suggested that short term immunity to norovirus exists as subjects symptomatic after an initial challenge remained asymptomatic after early, 6-14 weeks, re-challenge (Wyatt et al. 1974). Immunity was seen in a study by Johnson *et al* to last up to 6 months (Johnson et al. 1990). Longer term immunity was shown to be limited as patients who were symptomatic on initial challenge were also symptomatic on re-challenge 27-42 months later (Parrino et al. 1977). Therefore, immunity to norovirus infection is likely to last for between 6 months and 2 years. The duration acquired immunity protects against infection is complicated by the evolution of norovirus, as described in section 1.1.4. This evolution leads to antigenic variation which may limit the protection offered by acquired immunity.

Immunological responses have been measured in faeces and serum. In faeces the immune response detected has been shown to be associated with rises in interferon gamma and IL-2. These are seen in T helper 1 cell responses which are associated with intracellular pathogens

(Ko et al. 2006). This results in macrophage activation, B cell differentiation to IG1 synthesis and support for cytotoxic T lymphocytes. In addition faecal IgA responses are also elevated (Okhuysen et al. 1995). In studies of serum IgG responses > 4 fold increases are seen in response to norovirus infection (Lindesmith et al. 2005). Their ability to offer protection across the norovirus genogroups/genotypes is uncertain. It is likely some protection may be offered across a genogroup of norovirus (Matsui and Greenberg 2000). More recent studies have looked specifically at the GII.4 strain and the different variants which have emerged over recent years. These studies have shown that genetic changes associated with GII.4 strains produces variants which induce antibodies that bind poorly to different GII.4 viral variants (Lindesmith et al. 2008).

1.2 THE CURRENT STUDY

1.2.1 Background

Norovirus diagnosis is based on testing faecal samples and initially used electron microscopy (EM) (Kapikian 2000). EM only has an approximately 30% sensitivity and can only be used in centres with EM facilities, these are not common even in the more developed world. The detection of norovirus improved with the use of PCR and ELISA tests (Kageyama et al. 2003). PCR has become the gold standard test as it is the most sensitive test. The limitations of this method are based on its requirement for faecal samples, which in our experience can be difficult to collect, and the requirement for specific equipment and technical expertise to complete PCR. PCR is now a routine test in many clinical laboratories in the developed world. ELISA based testing offers a more low technology means of diagnosing norovirus infection. ELISA testing is therefore suitable for laboratories without PCR based testing, but has been limited by a lack of analytical sensitivity compared to PCR (Gray et al. 2007). Both PCR and ELISA based testing is commonly conducted on batches of samples to make testing more cost-effective. This can delay the time it takes for a result to be obtained, important in nosocomial outbreaks of infection given the short incubation time and high secondary attack rate.

1.2.2 Objectives

This study investigates new diagnostic tests which may improve norovirus testing with regard the limitations of current testing, namely: The need for faecal samples, the sensitivity of ELISAs and the costs and time associated with testing small batches of samples for norovirus. The process of investigating these new diagnostic tests also allows an assessment of the molecular epidemiology of norovirus in two areas, Merseyside, UK and Aracaju, Brazil. The specific aims are:

1- To evaluate the diagnostic accuracy of one step real time RT-PCR of oral samples to diagnose norovirus infection.

Norovirus testing currently requires the use of faecal samples to be tested, which can be difficult to collect. This is because faeces are traditionally collected in pots. Collecting faecal samples in pots in clinical environments can be difficult as diarrhoea is intermittent, may only occur for a short period of time and stool is produced without sufficient warning. Given norovirus is commonly associated with vomiting, which may contaminate the oral cavity, investigating if an oral mouthwash could be a suitable specimen to support the diagnosis of norovirus infection was conducted.

2- To investigate the sensitivity and specificity of a new ELISA, the RIDASCREEN ELISA (R-Biopharm).

Currently, the norovirus ELISA with the best analytical sensitivity is approximately 60% sensitive when compared to PCR (Gray et al. 2007). This has restricted the role of ELISA to a cheap, quick and, in terms of analytic sensitivity, an insensitive method of screening samples for norovirus infection. Given the low analytical sensitivity of the ELISAs, PCR testing is used in preference in many diagnostic laboratories. If the analytical sensitivity of ELISAs improved they could be used for testing individual patients and in molecular epidemiological studies. Given many centres are without the capacity for molecular diagnosis of norovirus there is a need for a more sensitive ELISAs. In those centres with PCR testing available ELISAs may offer a cheaper and quicker way of testing samples for norovirus infection prior to completing molecular testing. Therefore a newly developed commercially available ELISA was investigated for its sensitivity and specificity in comparison to both PCR and an established ELISA, the IDEIA norovirus ELISA. The sample set the ELISA is tested on is a cohort of children in Brazil with gastroenteritis.

3- To investigate the sensitivity and specificity of a new immunochromatographic test for norovirus, the RIDAQUICK norovirus test (R-Biopharm).

In many outbreaks of norovirus infection only a small number of samples are collected for testing. The use of PCR based testing can therefore be an inefficient means of reaching a diagnosis. Immunochromatographic (ICG) testing allows testing to occur both rapidly, in approximately 20 minutes, and on single samples. A new ICG test for norovirus, RIDAQUICK is investigated to allow consideration of this test as a rapid diagnostic test for norovirus. The sample set the ICT test is tested on is derived from a cohort of Brazilian children with gastroenteritis.

4- To investigate the molecular epidemiology of norovirus infection in Merseyside, UK, and in Aracaju, Brazil.

Studies into the molecular epidemiology of infectious diseases are carried out to provide information on the interaction of host/environment and the pathogen e.g. transmission routes. The intended outcome of understanding this interaction is to improve strategies which are used to prevent infections by a pathogen e.g. by reducing transmission rates. This study, therefore, investigates the molecular epidemiology of norovirus in two settings: hospitalised adults in Merseyside, UK and children with gastroenteritis in Brazil. In both these settings, improved norovirus prevention strategies would offer significant clinical benefits.

CHAPTER 2

An evaluation of the diagnostic accuracy of using oral samples to test for norovirus infection.

2.1 Introduction

Norovirus comprises a genetically diverse genus within the *Caliciviridae* family. Two main genogroups of norovirus (I and II), separated into over 30 genotypes, cause human infection (Green et al. 2000). They are the predominant causative agent of acute viral gastroenteritis in adults and one genotype, GII.4, is currently causing the majority of outbreaks of infection globally (Bull et al. 2006). Diarrhoea and vomiting are the principal clinical features of norovirus gastroenteritis (Lopman et al. 2004) and cause widespread contamination of the environment. This contamination, combined with the low infectious dose of norovirus (10-100 particles), results in institutional outbreaks of gastroenteritis, including in hospitals. These outbreaks are associated with a relatively mild morbidity and low mortality (Harris et al. 2008), but the economic impact on healthcare institutions is significant (Lopman et al. 2004). As norovirus has a short incubation period, approximately 24 hours, the prevention of norovirus outbreaks relies upon prompt diagnosis of infection. Whilst this can be carried out by assessing clinical features, rapid laboratory confirmation (or exclusion) of norovirus infection is likely to help rationalise the use of infection control resources. The detection of norovirus RNA in faeces by reverse transcriptase PCR (RT-PCR) is the current diagnostic test of choice, though ELISA and immunochromatographic tests are also available. These diagnostic methods rely on the collection of faecal samples. Collection of faeces can be problematic and cause diagnostic delay. Given vomiting often precedes diarrhoea in norovirus infection, leading to contamination of the oral cavity with norovirus, sampling from the oral cavity may allow the problem of collecting faecal samples to be negated. The evidence which supports such a strategy comes from two observations. Firstly, the diagnosis of norovirus using RT-PCR on vomit has been published (Dolin 2007). Secondly, an outbreak of norovirus infection in the family of a research worker at the University of

Liverpool, (to date unpublished), was used to complete a preliminary study into the oral detection of norovirus. This family outbreak demonstrated that norovirus could be detected for up to two weeks in mouthwash samples following infection. This family outbreak, followed by a study into the oral diagnosis of norovirus infection, is now presented.

2.1.1 Preliminary study into the oral diagnosis of norovirus infection

An outbreak of norovirus infection within the family of a researcher provided the opportunity to obtain faecal and oral samples (obtained by mouth washes and mouth swabs) during and following clinical illness. This allowed a preliminary study into the oral detection of norovirus to be conducted.

The study

The index case was a physiotherapist who worked at a hospital experiencing an outbreak of norovirus infection, and who was living in a family household of six. The index case returned home from work with diarrhoea, and the remainder of the household subsequently developed symptoms of gastroenteritis over the next 24-48 hours.

Faecal samples were collected into sterile universal containers (Sterilin, Stone, UK). Mouth swabs were obtained by rubbing a swab (Virocult: Medical Wire, Corsham, UK) over buccal, lingual and palatal surfaces. Mouthwashes were obtained by asking subjects to wash the mouth with sterile distilled water (3mls), the fluid then collected in sterile containers. Mouthwashes were collected from adults, and mouthswabs from the single infant in the study. Samples were collected in the morning prior to breakfast and were prepared for testing as follows: faecal samples by making a 10% suspension, mouth swabs by submersion in 1ml of PBS and mouthwash samples were taken directly for testing. Samples were vortexed before centrifuging a 1ml aliquot for 10 minutes at 14000rpm. Supernatant (500µl) from the centrifuged oral samples then underwent RNA extraction using QIAGEN RNeasy kits (QIAGEN: Sussex, UK). Extract was eluted into 30µl of RNAse free water and 3µl of RNA then subjected to RT-PCR using the method of Jiang *et al* (Jiang et al. 1999) in which a 319bp product is amplified from the RNA polymerase region. The product was detected by electrophoresing in a 2% agarose gel with ethidium bromide staining and viewed under ultraviolet (UV) light. Amplicons were purified using the QIAGEN gel purification kit and sequenced by Lark Technologies (Essex; UK). Negative controls were used in all RT-PCR runs. Faecal samples were processed as described by Dove *et al* (Dove et al. 2005).

The index case (case 1) developed diarrhoea without vomiting. Four family members (cases 2-5) developed diarrhoea and vomiting, and one member (case 6) suffered abdominal bloating without diarrhoea or vomiting. Four cases (2,3,5 and 6) had faecal samples collected at the onset of illness which underwent RT-PCR examination which were all positive for the presence of norovirus RNA. Norovirus RNA could be detected by RT-PCR of oral samples from all six cases, including cases 1 and 6 who had not vomited. Examination of serial oral fluids demonstrated that norovirus RNA could be detected in each case for 10-15 days following infection, after which it became undetectable (Figure 2.1). The nucleotide sequence of RT-PCR products detected in oral samples were identical to that seen in the faeces [(cases 2 and 3) data not shown]. The nucleotide sequence of the RT-PCR products from case 2 was a GII.3 norovirus (GenBank accession number AB236724). An asymptomatic staff member provided oral samples which were used as negative control samples.

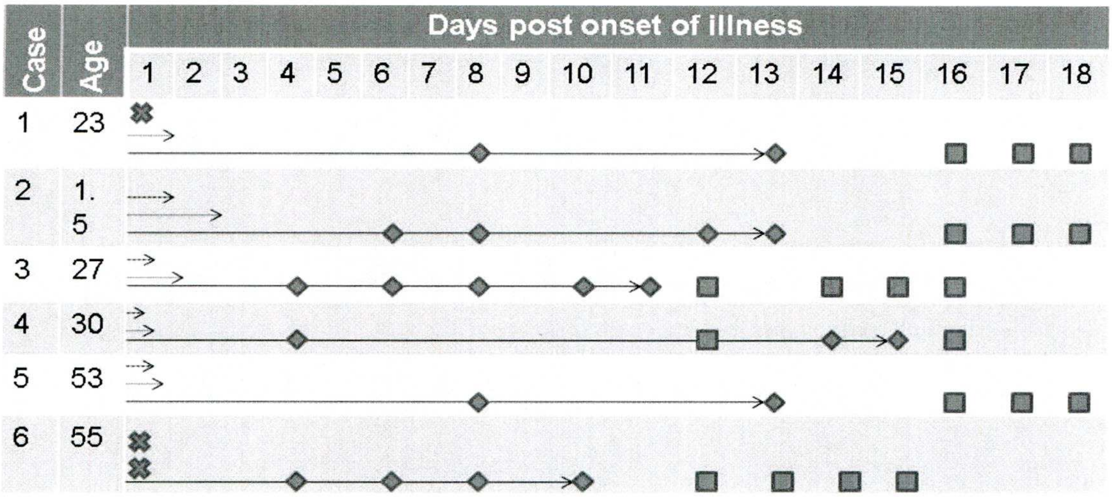


Figure 2.1: Temporal relationships between symptoms and oral detection of norovirus RNA following norovirus infection. For each case: top row = vomiting, middle row = diarrhoea, bottom row= detection of norovirus RNA in samples from oral cavity). ✖ = symptom not present. ◆ = day on which norovirus RNA detected from oral sample, ■ = day on which norovirus RNA not detected from oral sample.

2.1.2 Standards for Reporting of Diagnostic Accuracy criteria

This chapter presents the results of a study which is an evaluation of a diagnostic test. Evaluating diagnostic tests and subsequently publishing the tests characteristics is undertaken so others may make decisions about the clinical utility of that test. For others to make a decision about a test's clinical utility, they need complete information about the evaluation e.g. severity of disease and demographics of patients tested. A review in 1995 (Reid et al. 1995) of the reporting of evaluations of diagnostic tests found significant deficiencies. This led to the development of criteria for the evaluation of diagnostic tests, the Standards for Reporting of Diagnostic Accuracy criteria (STARD criteria), to be produced by the Cochrane Diagnostic and Screening Test Methods Working Group (Bossuyt et al. 2003). The STARD criteria are a checklist that it is recommended should be used in publishing the evaluation of diagnostic tests.

Presentation of the test evaluation according to the STARD criteria

The STARD criteria allow an evaluation of a diagnostic test to be presented in a clear and accessible format. Therefore, this chapter is presented in the format dictated by the STARD criteria. The table with the STARTD criteria (Table 2.1) allows the evaluation to be navigated easily and presentation of the evaluation in accordance with the criteria ensures all relevant details of the study are presented.

Table 2.1: STARD criteria reference table for the study: An evaluation of the diagnostic accuracy of real time reverse transcriptase PCR for norovirus in oral samples.

Section and Topic	**Item**		**Section**
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	2.2
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	2.2.1
METHODS			2.3
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	2.3.1.1
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	2.3.1.2
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	2.3.1.3
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	2.3.1.4
Test methods	7	The reference standard and its rationale.	2.3
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	2.3.1-2.3.3.4
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	2.3.4
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	2.3.5
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	2.3.6
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	2.3.7
	13	Methods for calculating test reproducibility, if done.	Not done
RESULTS			2.4
Participants	14	When study was performed, including beginning and end dates of recruitment.	2.4.1.1
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	2.4.1.2
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	2.4.1.3
Test results	17	Treatment administered in between study and reference test.	2.4.2.1
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	2.4.2.2
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	2.4.2.3
	20	Any adverse events from performing the index tests or the reference standard.	2.4.2.4
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	2.4.3.1
	22	How indeterminate results, missing data and outliers of the index tests were handled.	2.4.3.2
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	2.4.3.3
	24	Estimates of test reproducibility, if done.	Not done
DISCUSSION	25	Discuss the clinical applicability of the study findings.	2.5

Table reproduced from Bossuyt *et al* (Bossuyt et al. 2003)

2.2 Title /Abstract/Keywords

Title

An evaluation of the diagnostic accuracy of using oral samples to test for norovirus infection.

Abstract

Rapid diagnosis of norovirus infection may help in the control of nosocomial outbreaks. Laboratory diagnosis can be delayed while awaiting faecal samples for testing. An investigation was conducted into the use of oral samples to diagnose norovirus infection as these can be collected on demand. Real time reverse transcriptase PCR was carried out on 66 paired faecal and oral samples. Norovirus infection was confirmed in 59 faecal samples. Of these 59 patients, 14 (24% (95% C.I. 14-37%) were positive for norovirus in the oral samples. Oral sampling is not currently suitable for routine diagnosis of nosocomial norovirus infection.

Keywords

Norovirus; Oral; Diagnosis; Test; Sensitivity; Specificity

2.2.1 Aims

1- To evaluate the diagnostic accuracy of one step real time reverse transcriptase PCR of oral samples to diagnose norovirus infection.
2- To make an assessment of clinical factors which affect the sensitivity of one step real time reverse transcriptase PCR of oral samples to diagnose norovirus infection.

2.3 METHODS

2.3.1 PARTICIPANTS

2.3.1.1 Study population

Participants were recruited for the study from November 2008 until February 2009

Inclusion Criteria

- Vomiting and/or diarrhoea.
- Diarrhoea within the preceding 72hours.

Diarrhoea was defined as 1 or more episodes of loose stools.

Exclusion Criteria

- Another obvious cause of diarrhoea and/or vomiting.
- No age / sex / prior disease / prior treatment exclusion criteria used.
- Severe / life threatening or terminal illness or unable to consent.

Settings

Patients were identified within hospital wards from four NHS trusts (five hospitals) in the North West of England, UK.

- Royal Liverpool and Broadgreen University Hospital NHS Trust. Two study hospitals: The Royal Liverpool University Hospital (RH) and Broadgreen Hospital (BG), on separate sites. Liverpool, UK.
- Liverpool Heart and Chest Hospital NHS Trust. Liverpool Heart and Chest Hospital (CT) on the same site as Broadgreen Hospital. Liverpool, UK.
- Wirral University Teaching Hospital NHS Foundation Trust. Arrow Park Hospital (AP), Upton, Wirral, UK
- St Helens & Knowsley NHS Trust, Whiston Hospital (WH), Prescot, UK.

2.3.1.2 Participant recruitment

Patients were recruited based on their clinical symptoms, see section 2.3.1.1.

Ethics: Ethics approval was gained from the Bolton Research Ethics committee: REC No. 08/H1009/12, for full details see Appendix.

2.3.1.3 Participant sampling

Patients were identified for the study in two ways.

- Surveillance of laboratory requests for norovirus testing at RH by the principal investigator of the study, A Kirby.
- Reporting of suspected outbreaks of norovirus by medical and nursing staff, involved in hospital infection control, from each NHS trust, to the principal investigator of the study (A Kirby). An outbreak was not strictly defined. Local infection control staff defined outbreaks according to local policy.

Patients were clinically assessed by the principal investigator of the study at the bedside. If they met the eligibility criteria they were consented and entered the study. The oral sample would then be taken at the bedside by the principal investigator (A Kirby). The patient was asked to provide a faecal sample when possible. Oral sampling consisted of asking a patient to swill 3mls of sterile water around the mouth for 10 seconds with collection of the water in a sterile container. Samples were stored at 4^0C until extraction; this was carried out within 24 hours of sample collection.

2.3.1.4 Data collection

Data was collected prospectively.

2.3.2 Test methods

The choice of PCR method used in this evaluation to detect norovirus is a one step real time RT-PCR method using faecal samples. This method was used because it is the method routinely used in the clinical laboratory at the Royal Liverpool Hospital. This method is established and undergoes external evaluation.

2.3.2.1 Test under evaluation: One step real time RT-PCR of oral/faecal samples

One step real time RT-PCR: Principle of test

One step real time RT-PCR consists of the following stages. RNA extraction: RNA is extracted from the clinical samples to provide an RNA template for reverse transcription: RNA is reverse transcribed using a reverse transcription enzyme providing complementary DNA (cDNA) for the PCR step. PCR: cDNA is amplified using a DNA polymerase reaction to provide a product of a specific size. In real time PCR a probe with the potential to fluoresce is included in the PCR reaction mixture. This probe binds specifically to cDNA until cDNA is replicated by DNA polymerase. DNA polymerase removes the probe which then fluoresces. This fluorescence is detected and is proportional to the specific replication of the norovirus derived cDNA. One step PCR combines the RT and PCR mixture in the same tube as opposed to standard RT-PCR where RT and PCR are carried out as separate reactions.

One step real time RT-PCR: Test procedure

Preparation: Oral samples: Samples were collected by swilling 3mls of sterile water in the mouth. Samples were centrifuged at 14000rpm for 10 minutes and the supernatant used as described below. Faecal samples: Samples were prepared by diluting 100μl of liquid stool or 50-100mg of solid stool into 0.9ml of phosphate buffered saline (PBS). Samples were homogenised by mixing with a vortex. Samples were then centrifuged at 14000rpm for 10 minutes. The supernatant was used as described below.

The test: RNA extraction: This was undertaken using QIAGEN mini columns (QIAGEN, West Sussex, UK). 500µl of faecal/oral supernatant was mixed with 300µl of RLT buffer and 300µl of 70% ethanol. This mixture was then added to QIAGEN mini columns and centrifuged at 14000rpm. 700µl of RW1 wash buffer was then added to each column, followed by centrifugation at 14000rpm for 15 seconds. Next, 500µl of RPE buffer was added to the column, centrifuged at 14000rpm for 15 seconds before again adding 500µl of RPE buffer to each column and centrifuging at 14000rpm for 2 minutes. The tubes were then centrifuged without addition of any reagent to the columns for 2 minutes at 14000rpm. Finally, 45µl of high-performance liquid chromatography (HPLC) water was added directly onto the silica membrane of the mini columns, incubated at room temperature for 1 minute and centrifuged at 14000rpm for 1 minute. The flow though contained the extracted RNA and was stored at -70^{0}C until used for the PCR reaction.

One step real time RT-PCR: The PCR mixture was prepared and included:

Supermix (Invitrogen, Paisley, UK)	10µl
Express mix (Invitrogen)	2µl
Primer mix (Metabion, Martinsried, Germany)	1µl
Probe mix (Applied Biosystems, Foster City USA)	0.5µl
HPLC water	1.5µl
RNA extract	5µl
Total	20µl

The primer and probe mixes were made at 10μM concentrations.

Primers (Kageyama et al. 2003; Pusch et al. 2005)

Cog1-F	CGY TGG ATG CGN TTY CAT GA
GI_NV193as	CGT CCT TAG ACG CCA TCA TCA
Cog2-F	CAR GAR BCN ATG TTY AGR TGG ATG AG
Cog2-R	TCG ACG CCA TCT TCA TTC ACA

Probes (Kageyama et al. 2003)

GI_TM9-MGB-VIC	VIC-TGG ACA GGA GAT CGC-NFQ
Ring2	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ

The PCR reaction was carried out in a thermocycler (Light cycler: Roche 480), cycling conditions were 50^{0}C for 15 minutes, 95^{0}C for 2 minutes followed by 45 cycles of 95^{0}C for 15 seconds, 56^{0}C for 60 seconds and 72^{O}C for 1 second. Fluorescence was detected on channels FAM (GII) and HEX (GI).

Quality control: Test results were verified by the inclusion of positive and negative controls in each batch of samples.

2.3.3 ADDITIONAL TEST METHODS

2.3.3.1 Qualitative RT-PCR

Qualitative RT-PCR: Principle of test

PCR product is formed by RT and PCR steps as for one step PCR. PCR product formed is detected by a different method, PCR product being stained with ethidium bromide. This stained product is then run on an electrophoresis gel to separate out products according to their size. Products of the correct size, which vary according to the primers used, can be identified by examining the gels under UV light.

Qualitative RT-PCR: Test procedure

Preparation: See one step real time RT-PCR: test procedure.

The test: RNA extraction: See one step real time RT-PCR: test procedure.

Reverse transcription: 40μl of the RNA was denatured by placing it in a thermocycler (2720 Thermal Cycler, Applied BioSystems, Foster City, USA) at 95°C for 5 minutes. The RT reaction mixture was prepared which contained:

HPLC water	11μl
5x buffer (Invitrogen)	6μl
50mM $MgCl_2$ (Invitrogen)	7μl
20μM Random hexamers (6N Sigma, Dorset, UK)	1μl
10mM dNTPs (Invitrogen)	2μl
RNase inhibitor (Promega, Southampton, UK)	1μl
M-MLV reverse transcriptase (Invitrogen)	2μl

30μl of RT mix was added to the denatured sample, and then placed back into the thermocycler at 37°C for 1 hour, followed by 95°C for 5 minutes.

PCR: A 50μl PCR mixture was prepared for each sample and included:

HPLC H_2O	33.75μl
10X buffer (Invitrogen)	5.0μl
50mM MgCL2 (Invitrogen)	1.5μl
10mM dNTPs (Invitrogen)	1.0μl
10μM primers	3.0μl
5units/μL Taq polymerase (Invitrogen)	0.75μl
cDNA	5.0μl
Total	50μl

The PCR reaction was then undertaken in a 2720 Applied BioSystems Thermal Cycler under the following conditions: 1 Cycle for 3 min at 94°C, 40 cycles for 1 min at 94°C, 1 min at 50°C and 2 min at 72°C,1 cycle for 15 min at 72°C. Once the PCR reaction finished the PCR products were run on a 2% agarose gel (Bioline, London, UK) and stained with ethidium bromide. Agarose gel was prepared with 0.5XTBE (Tris base, boric acid and EDTA). After electrophoresis amplification products were visualized under UV light. Products co-migrating with the positive control with a product size of 330bp for GI and 344bp for GII virus were considered positive. The primers used were (Kojima et al. 2002):

G1SKF	CTG CCC GAA TTY GTA AAT GA
G1SKR	CCA ACC CAR CCA TTR TAC A
G2SKF	CNT GGG AGG GCG ATC GCA A
G2SKR	CCR CCN GCA TRH CCR TTR TAC AT
G2ALSKR	CCA CCA GCA TAT GAA TTG TAC AT

Quality control: Test results were verified by the inclusion of positive and negative controls in each batch of samples. In addition a 100bp lambda ladder (Invitrogen) was run on each electrophoresis gel.

2.3.3.2 DNA purification

Sequencing of PCR products was conducted where sufficient PCR product was produced. To allow sequencing the products from the PCR needed purifying, two methods were used to purify norovirus PCR product. Microspin columns were used if the 344bp product was the predominant product on the agarose gel. If multiple products were seen on the agarose gel the QIAquick method was used.

Illustra Microspin Columns (GE Healthcare, Buckinghamshire, UK): Principle of test

DNA is purified by the use of a resin. The resin allows gel filtration of an applied sample. Molecules larger than the largest pores in the resin e.g. DNA, do not penetrate the resin matrix and are the first molecules to elute from the resin allowing their preferential collection.

Illustra Microspin Columns (GE Healthcare): Test procedure

Preparation: Microspin columns were re-suspended by vortexing then centrifuging for one minute at 3000rpm.

The test: PCR product was pipetted on to the spin column membrane and the column centrifuged at 3000rpm for 2 minutes. Purified DNA was collected in an eppendorf tube.

Quality control: No specific quality control checks are in place for this procedure. Confirmation it has been successful is derived from the sequence data produced.

QIAquick Gel Extraction Kit (QIAGEN): Principle of test

The QIAquick columns contain a size fractionated silica membrane. When a sample is applied to the membrane impurities such as excess primers and agarose remain unbound. The correct chaotropic (nucleic acid denaturing agent) salt concentration (high) and pH (<7.5) ensure binding of DNA. Wash buffers are used to remove the chaotropic salts and DNA is eluted from the membrane using a non acidic low salt concentration solution e.g. water.

QIAquick Gel Extraction Kit (QIAGEN): Test procedure

Preparation: The PCR product was visualized using UV light in a dark room and cut from the agarose gel.

The test: The gel was weighed and 3 volumes of BufferQG were added to each 1 volume of gel. Excised gel fragments were dissolved by incubating for 10 minutes at 50°C. 1 gel volume of isopropanol was added to the sample mix which was added to the QIAquick column and centrifuged for 1 minute at 13000rpm. After centrifugation 750µl of Buffer PE was added to the QIAquick column and centrifuged for 1 minute at 13000rpm. Flow through was discarded and the column centrifuged (13000rpm) for an additional 1 minute. The DNA was then eluted by adding 50µl of distilled water to the centre of the membrane followed by centrifugation at 13000rpm for 1 minute.

Quality control: No specific quality control checks are in place for this procedure. Confirmation it has been successful is derived from the sequence data produced.

2.3.3.3 Sequencing of PCR products

All of the purified PCR products were sequenced by Cogenics (Essex, UK). Methods were requested from them which are now provided. DNA sequencing was performed using the Big

DyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). Sequencing reactions are analyzed on a 3730xl automated DNA sequencer (Applied Biosystems). The raw data is analyzed using ABI Prism® DNA Sequencing Analysis Software Version 5.0.

2.3.3.4 Analysing sequence data

The norovirus genome consists of three open reading frames, ORF 1/2/3. The Primers used in this study to generate sequence data were G1SKF, G1SKR, G2SKF, G2SKR and G2ALSKR (Kojima et al. 2002). These primers span the region at the 3 prime end of ORF1 and the 5 prime end of ORF2. The sequences were aligned by removing the ORF1 section. This was done by finding the ATG start codon of ORF2. An approximately 278bp region of ORF2, starting from the start codon, was selected for phylogenetic analysis. The section of sequence selected for analysis was based on quality of the sequence data. Software packages were used to edit the sequence obtained (BioEdit and SeqMan), align the sequences (EditSeq and Megalign) and produce phlyogenetic trees (CLUSTALW at Data Bank of Japan (DDBJ) [http://clustalw.ddbj.nig.ac.jp]. DDBJ aligns sequences using the neighbor joining (NJ) method. Sequences used in the phylogenetic analysis included representatives from the genotypes GI.1-14 and GII.1-19. Norovirus sequences were grouped as a distinct strain where the sequence nucleotide data was 100% similar. Percent similarity between norovirus sequences was calculated using Megalign computer software: sequence distances function.

2.3.4 Categorisation of test results

Faecal/oral samples: Only GII virus was detected in this study. The cut off for detection was a fluorescence over 3.7. The cut off was calculated with reference to the negative control fluorescence values. The mean fluorescence was 0.5 (0.05-2.2), with a standard deviation of 0.5. The cut off was therefore set above this value at 3.7, equal to the highest negative value plus 3 standard deviations.

2.3.5 Competencies of staff completing laboratory work

Laboratory testing was carried out by personnel with experience of norovirus diagnostics (L Ashton, Royal Liverpool University Hospital and A Kirby, Liverpool University).

2.3.6 Potential for result bias

Samples were processed and analysed without knowledge of clinical details.

2.3.7 Statistical methods

Statistical support was received from:

Stephen Taylor, Centre for Medical Statistics, Liverpool University; Steven Lane, Centre for Medical Statistics, Liverpool University; Brian Faragher, Liverpool School of Tropical Medicine, Liverpool University.

2.3.7.1 Sample size calculation

Samples size was calculated to see if the oral diagnosis of norovirus by PCR had a sensitivity compared to faecal PCR of > 50%. This value was decided upon as norovirus in hospitals commonly causes outbreaks of infection which affect multiple patients at the same time. The detection of norovirus in one patient is routinely used as evidence other patients with a norovirus syndromic presentation also have norovirus infection. The diagnosis of norovirus in a cohort of symptomatic patients can therefore be considered in terms of multiple tests on different patients as opposed to a single test on a single patient. Figure 2.2 shows how joint sensitivities can lead to an acceptable sensitivity of multiple tests despite a low sensitivity in an individual test.

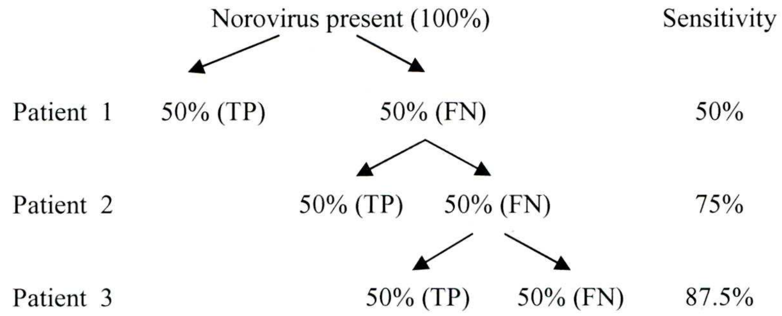


Figure 2.2: The effect of joining sensitivities of an oral norovirus test with a sensitivity of 50% per sample.

A sample size with a sensitivity lower bound confidence interval of >50% was therefore required (at a 95% confidence interval).

The formula for calculating sample size with a defined lower bound is given by:

$n = 1.96^{2} \times p(1-p) / (p-p_0)^2$

where: n= sample size, p= sensitivity of the test under evaluation, p_0= accepted lower bound of the confidence interval around this anticipated sensitivity value(p). If the test sensitivity is estimated at 63% and the lower bound of the tests C.I. is >50% the sample size can be calculated as below:

p=63%

p_0=52.42%

$n= 1.96^2 \times 0.63(1-0.63) / (0.63-0.5242)^2$

n=80

The sample size is therefore 80 cases of norovirus infection.

Examining previous nosocomial outbreaks identified norovirus in 50% of samples submitted to our laboratory (RH) for testing. Given this a sample of 160 is required to detect a sensitivity with a lower bound 95% confidence interval of >50%.

2.3.7.2 Methods for interpretation of results

Reporting of diagnostic accuracy: Results will be presented in a cross tabulation table as shown below (Table 2.2).

Table 2.2: Cross tabulation for presenting study results.

		Reference standard	
		+	-
Test under evaluation	+	True positives	False positives
	-	False negatives	True negatives

True positive (TP): A sample testing positive by the test under evaluation and the reference standard.

False positive (FP): A sample testing positive by the test under evaluation and negative by the reference standard.

False negative (FN): A sample testing negative by the test under evaluation and positive by the reference standard.

True negative (TN): A sample testing negative by the test under evaluation and the reference standard.

The reference standard in this study, faecal PCR, is used to define the presence of norovirus disease.

The following characteristics of the test/statistical tests will be presented

1. **Sensitivity=** True positives / True positives + False negatives
2. **Specificity=** True negatives / True negative + False positives

These values will be presented with 95% confidence interval

3. **Negative predictive value (NPV)**, based on a norovirus prevalence of 50%.

   NPV= True negatives/True negatives + False negatives

4. **Positive predictive value (PPV),** based on a norovirus prevalence of 50%.

   PPV=True positives/True positives + False positives

5. **McNemar`s test.** To analyse the statistical significance in the differences in the diagnostic accuracy of the oral norovirus test and the faecal norovirus test the McNemar`s test was used. This test was used as the data is categorical, summarised as a percentage and paired.

6. **T-test: Unpaired.** To analyse the statistical significance in the differences in the clinical features in patients with/without norovirus detected by the oral norovirus test the t-test was used (two sided). This test was used as the data is continuous and independent.
7. **Fishers exact test.** To analyse the statistical differences in the detection of norovirus in oral samples in those with/without vomiting and diarrhoea Fishers exact test was used (two sided). This test was used as the data is categorical, expressed as a percentage and independent.

2.3.8 Test reproducibility

Test reproducibility was not assessed in this study.

2.4 RESULTS

2.4.1 PARTICIPANTS

2.4.1.1 Timing of participant recruitment

Participants were recruited for the study from November 2008 until February 2009.

2.4.1.2 Clinical and demographic characteristics

Participants

- Total Number: 100.
- Mean age: 77 years (range 24-97).
- Sex: 35 male and 65 female.

Locations of participants: Hospital / Ward (number of participants)

- Arrow Park Hospital (55): AMAU (2), Ward 23 (2), Ward 24 (6), Ward 25 (1), Ward 26 (5), Ward 27 (9), Ward 32 (2), Ward 33 (6), Ward 37 (10), Ward 38 (4), Ward 43 (8).
- Broadgreen Hospital (15): Ward 11 (5), Ward 5 (8), Ward 7 (2).
- Liverpool Heart and Lung Hospital (4): Ward A (4).
- Whiston Hospital (16): Ward B2 (3), Ward C3 (8), Ward F1 (5).
- Royal Liverpool Hospital (10), Ward 3A (1), Ward 3X (1), Ward 5B (1), Ward 8A (2), Ward 8B (1), Ward 8MCU (1), Ward 9X (3).

2.4.1.3 Investigative strategy

All patients meeting the inclusion criteria and who gave consent underwent oral sampling The laboratory investigations consisted of one step real time RT-PCR followed by (if positive), qualitative PCR. If positive by qualitative PCR with sufficient PCR product, DNA sequencing was then conducted. Of those patients who underwent oral sampling 66 provided faecal samples which were tested by one step real time RT-PCR. A representative selection, with reference to study location, of these underwent qualitative PCR and sequencing.

2.4.2 TEST RESULTS

2.4.2.1 Treatment administered in between study and reference test.

No specific anti-norovirus treatment was administered between oral and faecal sampling.

2.4.2.2 Disease severity in participants

Disease severity can affect a tests sensitivity and specificity. The clinical factors most likely to affect test sensitivity and specificity were considered to be duration of infection at testing and time since the last episode of diarrhoea/vomiting. These clinical factors were recorded at the time of patient sampling. The clinical severity outside these factors was not analysed given the heterogeneous co-morbidities in these hospitalised patients. Vomiting was present in 58% of participants, and the mean time since onset of vomiting to sampling was 2.9 days (0-23 days). Diarrhoea was present in 96% of participants and the mean time since onset of diarrhoea to sampling was 2.3 days (0-23). Time since last vomit was a mean of 21 hours (0-7days) and time since last diarrhoea was a mean of 13 hours (0-3 days). Analysis of these variables showed that they were associated with the detection of norovirus in the oral cavity as follows. The detection of norovirus in the oral cavity was associated with shorter durations of vomiting and diarrhoea and shorter times since the last episode of diarrhoea and vomiting, at the time of oral sampling (Table 2.3).

Table 2.3: Impact of clinical features associated with norovirus on the results of the oral diagnostic test for norovirus and associated p values.

	Real Time PCR		Qualitative PCR		Norovirus sequence		P value
	+ (14)	- (45)	+ (6)	- (53)	+ (4)	- (55)	
Vomiting present	12/14	26/45	5/6	33/53	4/4	34/55	0.1[1]
Mean duration of time since onset of vomiting (days (S.D))	0.7(0.8)	3.81(5.0)	.	.	.	.	0.03
Mean duration of time since onset of vomiting (days(S.D))	.	.	0.8(1.1)	3.1(4.2)	.	.	0.27
Mean duration of time since onset of vomiting (days(S.D))	.	.	.	.	1.3 (1.2)	3.0(4.6)	0.74
Mean time since last vomit (days(S.D))	0.4(0.6)	1.8(1.6)	.	.	.	.	0.006
Mean time since last vomit (days (S.D))	.	.	0.56(0.8)	1.5(1.6)	.	.	0.2
Mean time since last vomit (days (S.D))	.	.	.	.	0.6 (0.9)	1.5(1.5)	0.30
Diarrhoea present	13/14	45/45	5/6	53/53	3/4	55/55	0.23[1]
Mean duration of time since onset of diarrhoea (days(S.D))	0.7(0.8)	3.0(3.9)	.	.	.	.	0.046
Mean duration of time since onset of diarrhoea (days(S.D))	.	.	0.8(1.1)	2.7(3.7)	.	.	0.27
Mean duration of time since onset of diarrhoea (days(S.D))	.	.	.	.	1.3 (1.2)	2.6(3.7)	0.56
Mean duration of time since last diarrhoeal episode (days(S.D))	0.2(0.3)	0.8(0.7)	.	.	.	.	0.008
Mean duration of time since last diarrhoeal episode (days (S.D))	.	.	0.2(0.2)	0.7(0.7)	.	.	0.18
Mean duration of time since last diarrhoeal episode (days(S.D))	.	.	.	.	0.3(0.3)	0.6(0.7)	0.35

[1] Denotes the p value relates to real time PCR results. Durations of symptoms are those at the time of oral sampling. Vomiting/diarrhoea present = symptom having occurred by the time of oral sampling.

2.4.2.3 Cross-tabulation of results

In total 66 patients met the inclusion criteria and submitted faecal and oral samples for norovirus testing. Within these patients 59 had norovirus detected in faeces. The sensitivity of one-step real time RT-PCR, compared to faecal PCR, for the detection of norovirus in oral samples was 24% at 100% specificity (Table 2.4).

Table 2.4: Sensitivity and specificity of the oral diagnosis of norovirus by real time reverse transcriptase PCR in comparison to faecal diagnosis

	Faeces one step real time RT-PCR	
Oral one step real time PCR	+	-
+	14	0
-	45	7
	59	7
	Sensitivity 24%	Specificity 100%

2.4.2.4 Adverse events from testing

No adverse events were noted from the testing.

2.4.3 ESTIMATES

2.4.3.1 Estimates of diagnostic accuracy and predictive values

The 95% confidence intervals surrounding the sensitivity and specificity of the oral sampling (section 2.4.2.3) for the detection of norovirus are 14-37% and 56-100% respectively. The oral sampling results had a high positive predictive value (100%) but a low negative predictive (56%) (Table 2.5).

Table 2.5: Estimates of the diagnostic accuracy associated with oral norovirus testing.

[1] PPV and NPV calculated assuming a norovirus prevalence of 50%.

Test	Sensitivity	Sensitivity 95% C.I	Specificity	Specificity 95% C.I	PPV[1]	NPV[1]
Oral real time RT-PCR	24%	14-37%	100%	56-100%	100%	56%

2.4.3.2 Handling of indeterminate results, missing results and outliers

Results were classified as positive/negative with no equivocal range. Faecal samples were not collected from a number of participants. These patients were excluded from the main analysis but discussed in section 2.4.3.3.

2.4.3.3 Estimates of variability of diagnostic accuracy between subgroups of participants

The sensitivity and specificity of norovirus testing was assessed where oral diagnosis was based on oral qualitative PCR and on confirmation of norovirus sequence obtained from the PCR products prepared from the samples obtained from the oral cavity (subsequently referred to as oral sequencing samples). The sensitivity of these methods was below that of the real time RT-PCR at 11% (qualitative PCR) and 7% (norovirus sequence confirmation) (Table 2.6).

Table 2.6: The sensitivity and specificity of norovirus testing comparing oral qualitative RT-PCR and oral sequencing vs. faecal real time RT-PCR.

	Oral qualitative	Oral sequencing
Sensitivity: True positives/All positives (%)	6/59 (11%)	4/59 (7%)
Specificity: True negatives/All negatives (%)	7/7 (100%)	7/7 (100%)

Contribution of sequencing data to the validity of the results

A total of 7 patients of the 100 sampled had norovirus sequence obtained from the PCR products prepared from these patients oral samples. Within these 7, there were 5 where the sequence was identical to either the paired faecal sample, or to the strain of norovirus detected within that study location at the same time. The sequence data from 2 samples could be confirmed as norovirus but was not of sufficient quality to compare it to other norovirus sequences. The sequence data associated with this study are presented in detail in Chapter Four.

No faecal sample group

There were 34 patients who met the study inclusion criteria, had samples from the oral cavity collected but a faecal sample was not submitted to the study. Within this group 30 were from outbreaks of gastroenteritis that were confirmed as being caused by norovirus. This group was likely to have a high incidence of norovirus infection. This is because of 62 patients who did provide faecal samples and were from outbreaks of gastroenteritis confirmed as being caused by norovirus; only 3 were negative for norovirus infection. The sensitivity of oral PCR in patients without faecal samples but from within norovirus outbreaks was 27% (8/30). Qualitative PCR was positive in 3 of these 8 samples, with all 3 having norovirus sequence obtained by sequencing the PCR product. Of these 3 sequences 2 were identical to faecal samples from the corresponding outbreak and one was confirmed as norovirus but the sequence quality was not of sufficient quality to compare to other sequences from the study.

2.4.3.4 Estimates of test reproducibility

Estimates of test reproducibility were not undertaken.

2.5 Discussion

The sensitivity of diagnosing norovirus infection in oral samples by real time RT-PCR was found to be 24%; by qualitative PCR 11% and by norovirus sequence confirmation 7%. The differences in sensitivities may be to be due to low levels of virus in the oral cavity and the superior sensitivity of real time RT-PCR compared to qualitative PCR (Pang et al. 2004; Akihara et al. 2005; Gunson et al. 2006; Sun et al. 2008). Whilst recruitment to this study was not complete, the confidence interval around the estimate of the tests sensitivity is 14-37%. This sensitivity is below what is likely to be clinically useful for the detection of nosocomial norovirus infection. It may though still be useful in situations where faecal samples are difficult to collect e.g. community outbreaks of gastroenteritis, or where samples can be collected soon after vomiting. The findings of this study do not match the successful detection of norovirus in the family outbreak described in section 2.11. The reasons for this are unclear, but may include:

- Different study populations; in the hospital study participants were predominately elderly, and all with other co-morbidities. This may have resulted in an impaired ability to produce samples with an equivalent quantity of norovirus RNA to participants in the family study.
- The samples in this study were collected at different times throughout the day. In the family outbreak they were collected early in the morning. There may be human characteristics which cause the oral cavity to collect norovirus RNA early in the morning. A possible cause could be nocturnal subclinical aspiration of gastric contents. This would require further work to confirm.

- The virus was different, in this study it was a GII.4 virus, in the family outbreak study it was a GII.3 virus. There is no obvious biological process that supports this affecting the detection of norovirus in the oral cavity.
- Hospitalised patients were supplied with drinking water when they had gastroenteritis. This regular provision of drinking of water may have led to decontamination of the oral cavity.
- The detection of norovirus in faeces may have represented clinical false positives (asymptomatic norovirus shedding). This may have resulted in underestimating the sensitivity of the oral detection of norovirus.

The detection of norovirus in this study was inversely associated with the duration of vomiting ($p = 0.03$) and diarrhoea ($p = 0.046$) at sampling. It was associated with the presence of vomiting ($p = 0.1$) and inversely associated with the time since the last vomit ($p = 0.006$) and diarrhoea ($p = 0.008$).

2.6 Limitations

- The sample size was not reached; this reduced the precision of the estimate of the diagnostic accuracy of the test.
- The difference in the sensitivity of oral samples to diagnose norovirus infection between the family outbreak and hospital study was not resolved.
- It was not possible to assign a duration of diarrhoea/vomiting in those patients without these symptoms. This may have affected the estimates these symptoms had on the sensitivity of the oral detection of norovirus.

2.7 Further work

The current diagnosis of norovirus requires the use of a faecal sample. Further studies should investigate diagnostic methods which do not require these samples which can be difficult to collect. Possible strategies could be to devise clinical criteria for the diagnosis of hospital outbreaks of norovirus infection. These criteria would be analogous to the Kaplan criteria used in community outbreaks of gastroenteritis (Kaplan et al. 1982). Another strategy could be swabbing of vomit/faecal samples which were not collectable in pots.

2.8 Conclusions

Oral sampling is unlikely to reliably allow a correct diagnosis of norovirus to be made in nosocomial outbreaks of gastroenteritis. New approaches are still required to improve the rapid diagnosis of norovirus infection which do not rely on the collection of faecal samples.

CHAPTER 3

An evaluation of the diagnostic accuracy of the 3rd generation RIDASCREEN norovirus ELISA and the RIDAQUICK immunochromatographic norovirus test, including a comparison of both to the second generation IDEIA norovirus ELISA.

3.1 Introduction

Gastroenteritis causes significant morbidity and mortality in children in the developing world (Bryce et al. 2005). Viruses including rotavirus, astrovirus, adenovirus 40/41, sapovirus and norovirus account for a large proportion of this disease burden (Al-Mashhadani et al. 2008; Bucardo et al. 2008; Koopmans 2008). Norovirus, as a cause of gastroenteritis, was discovered in 1972, but its role in paediatric gastroenteritis has only recently begun to be appreciated. To more fully understand the contribution of norovirus to childhood mortality and morbidity requires detailed clinical and epidemiological studies. An integral component of such studies is the diagnostic tests used, with the current gold standard diagnostic technique for norovirus being PCR. Given norovirus is excreted for a prolonged period of time, as detected by PCR, following symptomatic infection (approximately 4 weeks), some investigators consider that PCR based diagnostics are too sensitive and unable to distinguish current from past infection (Gallimore et al. 2004; Atmar et al. 2008; Phillips et al. 2009). This diagnostic challenge is also seen in rotavirus gastroenteritis, where ELISA based testing is used to diagnose infection (Phillips et al. 2009). Diagnosis of norovirus infection is also made difficult by the high degree of genetic variation seen in norovirus. Norovirus is a single-stranded RNA virus and possesses two genogroups which are associated with human infection, genogroup I and II (GI and GII). Within these genogroups are over 30 genotypes (Green et al. 2000). This genetic variation makes diagnosis of norovirus difficult. ELISAs for the diagnosis of norovirus, previously evaluated in the literature, have proven to have moderate analytical sensitivity for the detection of norovirus GII.4 strain, the most common genotype (approximately 66% (Gray et al. 2007)). Their sensitivity has been less for non GII.4 strains (Gray et al. 2007). Two new immunological tests have recently become available for the detection of norovirus. One is an ELISA-based assay, and another an immunochromatographic (ICG) test. These tests have been developed with the aim of being more sensitive than current immunological norovirus tests. The ICG test should also allow more rapid diagnosis of norovirus. This would be particularly useful as it may facilitate studies which would rely on rapid diagnosis of norovirus, e.g. studies into the treatment of norovirus infection in children attending hospital with gastroenteritis. An evaluation of the new norovirus ELISA (RIDASCREEN® 3rd generation norovirus ELISA, R-Biopharm, Darmstadt, Germany) and the new norovirus ICG test (RIDAQUICK® norovirus ICG test, R-Biopharm) was therefore undertaken. These two assays were compared to a PCR based test to estimate their diagnostic accuracy. In addition, the tests underwent a head to head comparison to the ELISA test that had the best sensitivity in published evaluations, the IDEIA™ ELISA (Oxoid, Ely, United Kingdom). The results are used to assess how useful the tests are, both for diagnosis of norovirus and for undertaking epidemiological surveys for norovirus.

3.1.1 Background to norovirus immunological testing

Norovirus remains a non-culturable virus (Duizer et al. 2004). Despite one publication reporting successful culture of norovirus using a physiologically relevant 3-dimensional, organoid model of human small intestinal epithelium (Straub et al. 2007), this has not been repeated in other laboratories (personal communication: Koopmans, M. Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands). ELISA based diagnostics for norovirus were therefore difficult to produce until a Baculovirus expression system was developed to allow the production of norovirus antigens (Jiang et al. 1992). The Baculovirus expression system is based on the introduction of a foreign gene into a region of the viral genome that is nonessential for viral replication. This is carried out using a transfer vector containing the target gene. The resulting recombinant Baculovirus encoding the introduced protein gene can be expressed in cultured insect cells. Using the norovirus like particles that were produced by this system, which had antigenic properties similar to norovirus, monoclonal antibodies (MAbs) and polyclonal antibodies were produced which could be used in diagnostic ELISAs. Evaluations of the first norovirus ELISAs were first published in 2002. There were two ELISAs produced at this time; the SRSV (II)-AD kit, (Denka Seiken Co. Ltd. Tokyo, Japan), and the IDEIA NLV kit, (DakoCytomation Ltd. Ely, United Kingdom)(Uchino et al. 2002; Yamagami et al. 2002; Richards et al. 2003). Both kits used MAbs with the ability to bind to genogroup I and II noroviruses. The Denka kit was reported to have a sensitivity and specificity of 68% and 76 % respectively (Uchino et al. 2002; Yamagami et al. 2002). The Dako kit reported a sensitivity and specificity of 56% and 98%, respectively (Richards et al. 2003). More recently, two different norovirus ELISAs became commercially available: The second generation RIDASCREEN ELISA and the second generation IDEIA ELISA (Schmid et al. 2004). These ELISAs were repeatedly evaluated but publications reported variable sensitivities and specificities (de Bruin et al. 2006; Okitsu-Negishi et al. 2006; Castriciano et al. 2007; Wilhelmi de Cal et al. 2007). This may have been attributable to either differing samples being tested or differing reference standards. This led to a multicentre evaluation of the RIDASCREEN ELISA and the IDEIA ELISA in 2007 (Gray et al. 2007). This is the most definitive evaluation to date of norovirus ELISAs and is therefore now presented in detail. The study consisted of six European centres' that evaluated the ELISAs in comparison to each institutions established PCR test. The sensitivities and specificities of the IDEIA ELISA were 58.9% and 93.9%, and for the RIDASCREEN ELISA were 43.8% and 96.4% respectively (Table 3.01). This evaluation also carried out a further analysis on a sub selection of samples which gave false positive samples by ELISA. This further analysis was conducted on samples in which a non norovirus gastrointestinal pathogen was identified. Of the ten false positive reactions investigated seven were confirmed positive for norovirus by a secondary PCR which used a different primer set (Table 3.02). This suggests the analytical specificities reported in the study by Gray et al, although already high (88-97%), were underestimates. There was also variation between the assays in terms of the genotypes that were detected. Where there was a statistical difference in the sensitivity for a specific genotype, the IDEIA ELISA was always more sensitive. A statistical difference was seen for genotypes GI.2/6 and GII.1/3/7 (Table 3.03).

Table 3.01: Sensitivities and specificities of the IDEIA norovirus and RIDASCREEN norovirus ELISAs compared with RT-PCR in a European multicentre evaluation (Gray et al. 2007).

		IDEIA Norovirus				RIDASCREEN Norovirus			
Country	No. of samples	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI
France	320	61	55-68	94	87-97	40	34-47	95.96	90-98
Germany	97	67	54-77	90	75-97	37	26-49	83.33	66-93
Italy	461	47	41-53	88	83-92	42	36-48	96.90	94-98
The Netherlands	414	67	62-73	93	87-96	36	31-42	92.00	87-95
Spain	425	76	69-82	98	95-99	70	62-76	99.20	97-100
United Kingdom	474	45	39-51	97	93-98	40	34-46	97.61	95-99
All	2,191	59	56-62	94	92-95	44	41-47	96.37	95-97

Table reproduced from Gray *et al* (Gray et al. 2007)

TABLE 3.02: Nonspecific reactivity detected with the norovirus EIAs and results of RT-PCR tests to confirm the presence of norovirus from the norovirus ELISA evaluation by Gray *et al* 2007.

Organism	No. of samples			
	Positive for organism	Positive by IDEIA EIA	Positive by RIDASCREEN EIA	Confirmed positive by PCR
Astrovirus	28	1	0	1
C. difficile	18	1	0	0
Enteric adenovirus	22	2	0	1
Rotavirus	61	2	1	2
Sapovirus	15	3	0	3

Table reproduced from Gray *et al* (Gray et al. 2007).

Table 3.03: Strain specific sensitivities of the IDEIA norovirus and RIDASCREEN norovirus ELISAs compared with RT-PCR in a European multicentre evaluation (Gray et al. 2007).

Geno-group	No. of positive samples	IDEIA Norovirus		RIDASCREEN Norovirus		*P* value
		No. (%) of samples in which genotype was detected	95% CI	No. (%) of samples in which genotype was detected	95% CI	
GI-1	5	4 (80.00)	37.55-96.36	3 (60.00)	23.1-88.24	0.49
GI-2	13	11 (84.62)	57.77-95.67	2 (15.38)	4.33-42.23	0.0002
GI-3	28	12 (42.86)	26.51-60.93	9 (32.14)	17.9-50.66	0.4
GI-4	2	2 (100.00)	34.24-100.0	0 (0.00)	0.00-65.76	0.3
GI-5	8	3 (37.50)	13.68-69.43	0 (0.00)	0.00-32.44	0.2
GI-6	7	5 (71.43)	35.89-91.78	0 (0.00)	0.00-35.43	0.02
GI-7	1	0 (0.00)	0.00-79.35	0 (0.00)	0.00-79.35	>0.5
GII-1	8	7 (87.50)	52.91-97.76	0 (0.00)	0.00-32.44	0.0024
GII-2	16	8 (50.00)	28.00-72.00	4 (25.00)	10.2-49.50	0.2
GII-3	52	30 (57.69)	44.19-70.13	11 (21.15)	12.2-34.03	0.0003
GII-4	301	203 (67.44)	61.96-72.49	186 (61.79)	56.2-67.10	0.17
GII-5	6	2 (33.33)	9.68-70.00	1 (16.67)	3.01-56.35	>0.5
GII-6	9	2 (22.22)	6.32-54.74	0 (0.00)	0.00-29.91	0.4
GII-7	29	20 (68.97)	50.77-82.72	5 (17.24)	7.60-34.55	0.002
GII-8	1	0 (0.00)	0.00-79.35	0 (0.00)	0.00-79.35	>0.5
GIV-1	4	0 (0.00)	0.00-48.99	0 (0.00)	0.00-48.99	>0.5

Table reproduced from Gray *et al* (Gray et al. 2007)

The RIDASCREEN ELISA has been replaced by a third generation ELISA which is now commercially available. No peer reviewed publications using this ELISA have been identified, and available information on its performance is provided by the manufacturer, R-Biopharm, who report a sensitivity of 83% and a specificity of 100%. This is based on a retrospective assessment of 183 samples collected during a norovirus season (2004/2005) with 100 samples positive for norovirus by RT-PCR and 83 negative by RT-PCR. immunochromatographic testing is an alternative to ELISA testing, and is based on similar antigen-antibody interactions. The first publication describing the development of an ICG test for norovirus was in 2003 (Okame et al. 2003). The evaluations conducted since are shown below (Table 3.04). These ICG tests were produced for the detection of GII.3/4 norovirus (NV IC-1 Stick Kit and Takanashi Kit) and GI/GII norovirus (Quick Ex-Norovirus Kit).

Table 3.04: Comparison of rapid immunological tests for norovirus (Mutoh et al. 2009).

	Sensitivity (%)	Specificity (%)	Study/No. of samples
ICG kits			
NV IC-1 Stick kit (Immuno-Probe, Saitama, Japan)	73.7	100	(Nguyen et al. 2007) /n=14
NV IC-1 Stick kit (Immuno-Probe, Saitama, Japan)	78.9	96.4	(Khamrin et al. 2008)/ n=100
Quick Ex-Norovirus kit (Denka Seiken, Tokyo, Japan)	73.6	98.9	Manufacturer
Quick Ex-Norovirus kit (Denka Seiken, Tokyo, Japan)	54.5	93	Mutoh *et al* (Mutoh et al. 2009)/ n=26
Kit by Takanashi *et al.*	69.8	93.7	Takanashi (Takanashi et al. 2008) /n=43

Table reproduced from Mutoh *et al* (Mutoh et al. 2009)

More recently the RIDAQUICK norovirus ICG test has become commercially available. This contains both G1 and G2 antibodies used as capture antibodies and are the same antibodies used in the RIDASCREEN norovirus ELISA. Sensitivities and specificities reported by the manufacturer of RIDAQUICK are 86% and 100% respectfully. This reported sensitivity is comparable to, and in fact slightly higher than, the RIDASCREEN ELISA.

3.1.2 Standards for Reporting of Diagnostic Accuracy criteria

The STARD criteria (Chapter 2, 2.1.2) for the reporting of investigations into diagnostic accuracy are followed in the reporting this study (Table 3.05).

3.1.3 ACCE criteria for evaluating diagnostic tests

This study will assess how useful the tests are for diagnosis of norovirus and for undertaking epidemiological surveys for norovirus. The diagnostic assessment should not be made solely on its diagnostic accuracy but should be made in terms of the tests clinical utility. For example, a diagnostic test with perfect analytical sensitivity and specificity but with no clinical utility would be of limited clinical use. The importance of the clinical utility of a test is being increasingly recognised and is now an area targeted by a specific funding category offered by the National Institute of Health Research in the UK (NIHR UK: Health Technology Assessment programme: Diagnostic tests and test technologies). One structured format of assessing a test's overall value is by following a framework called ACCE. This assesses tests in terms of their *A*nalytic validity, *C*linical Validity, *C*linical Utility, and *E*thical aspects. These criteria were developed by geneticists keen to rigorously evaluate new genetic tests. As such they were not specific for microbiological tests. These criteria have therefore been adapted for this dissertation (Table 3.06) to allow an evaluation of microbiological tests. These criteria are used to ensure the discussion covers the important points of the diagnostic tests. The full ACCE review of the evaluated tests is presented in the appendix.

Table 3.06: ACCE criteria for the evaluation of a diagnostic test.

Disorder/ Assay	Is an infectious organism, or a specific condition caused by an unknown infectious organism, being investigated?
	What is the infectious organism or condition being studied?
	What is the history, clinical findings and prognoses of this disease?
	What is the clinical and molecular epidemiology of this disease? Are there public health/political implications?
	What therapy is available? Is empirical therapy available? What are the side effects of therapy?
	What is the clinical setting in which the test is to be performed?
	What microbiological assays / non microbiological investigations are associated with this disorder? Which are you evaluating?
	How long does the assay take? How does this relate to the time course of the infection and other diagnostic assays?
	What time period in relation to an illness is the test relevant to?
	Are the assays used for screening/diagnostic purposes? Are the assays useful to rule in/rule out a condition?
	Is the assay completed within the same assay as others? Is the assay completed at the same time as other assays (parallel) or on the basis of other results (series)? Does the result lead to further assays/investigations being completed?
	What is the laboratory setting in which the assay is to be performed?
	Are there developments visible in the future (diagnostic, therapeutic or preventative0 that will affect the durability of the implications of the ACCE assessment? Is an ACCE analysis for this test a priority?
	Using the information obtained by answering the above define the clinical care pathway this assay fits into.
Analytic Validity	Is the assay qualitative or quantitative?
	How often is the test positive/negative when the infectious organism is present (analytic sensitivity) / absent (analytic specificity)?
	Is an internal quality control program defined and externally monitored?
	Have repeated measurements been made on specimens (e.g. positive/negative controls)?
	What is the within- and between-laboratory precision?
	If appropriate, how is confirmatory testing performed to resolve false positive results, how long does this take?
	What range/quality of patient specimens has been tested?
	How often does the test fail to give a useable result?
	How similar are results obtained in multiple laboratories using the same, or different technology?
Clinical Validity	How often is the test positive/negative when the infectious disease is present (clinical sensitivity) / absent (clinical specificity)?
	What is the prevalence of the disorder in this setting? What are the positive and negative predictive values?
	Are there methods to resolve clinical false positive results in a timely manner?
	Has the test been adequately validated on all populations to which it may be offered?
	What are the relationship between presence of organism and disease (e.g. colonisers/obligate pathogens)?
	What affects the relationship between organism and disease (e.g. immunosuppression, prosthetic material)?
Clinical Utility	What is the impact of a positive (or negative) test on patient care?
	What is the impact on the prevention of spread of infection?
	Is there an effective treatment or other measurable benefit? Is there general access to that treatment or benefit?
	Is the test being offered to a socially vulnerable population?
	What quality assurance measures are in place?
	What health risks can be identified for the intervention?
	What are the economic costs associated with testing and what are the economic benefits resulting from testing?
	What are the results of pilot trials?
	What facilities/personnel are available or easily put in place?
	Are there informed consent requirements?
	What methods exist for long term monitoring?
	What guidelines have been developed for evaluating clinical care pathway performance?
ELSI	What is known about transmission of infection, stigmatization, discrimination, confidentiality and personal/family social issues?
	Are there legal issues regarding consent, ownership of data and/or samples, patents, licensing, proprietary testing, obligation to disclose, or reporting requirements?
	What safeguards have been described and are these safeguards in place and effective?

Table adapted from Haddow JE and Palomaki GE (Haddow and Palomaki 2004).

3.1.4 STARD criteria reference table

This chapter presents the results of a study which is an evaluation of a diagnostic test. Evaluating diagnostic tests and subsequently publishing the tests characteristics is undertaken so others may make decisions about the clinical utility of that test. For others to make a decision about a test's clinical utility, they need complete information about the evaluation e.g. severity of disease and demographics of patients tested. A review in 1995 (Reid et al. 1995) of the reporting of evaluations of diagnostic tests found significant deficiencies. This led to the development of criteria for the evaluation of diagnostic tests, the Standards for Reporting of Diagnostic Accuracy criteria (STARD criteria), to be produced by the Cochrane Diagnostic and Screening Test Methods Working Group (Bossuyt et al. 2003). The STARD criteria are a checklist that it is recommended should be used in publishing the evaluation of diagnostic tests.

Presentation of the test evaluation according to the STARD criteria

The STARD criteria allow an evaluation of a diagnostic test to be presented in a clear and accessible format. Therefore, this chapter is presented in the format dictated by the STARD criteria. The table with the STARTD criteria (Table 3.05) allows the evaluation to be navigated easily and presentation of the evaluation in accordance with the criteria ensures details of the study are comprehensively presented.

Table 3.05: STARD criteria reference: Evaluation of the diagnostic accuracy of the 3rd generation RIDASCREEN and RIDAQUICK norovirus tests.

Section and Topic	**Item**		**Section**
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	3.2
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	3.2.1
METHODS			3.3
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	3.3.1.1
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	3.3.1.1
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	3.3.1.1
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	3.3.1.1
Test methods	7	The reference standard and its rationale.	3.3.2
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	3.3.2.1/2/3
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	3.3.2.4
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	3.3.2.5
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	3.3.2.6
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	3.3.3
	13	Methods for calculating test reproducibility, if done.	3.3.3
RESULTS			3.4
Participants	14	When study was performed, including beginning and end dates of recruitment.	3.4.1.1
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	3.4.1.2
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	3.4.1.3
Test results	17	Time interval between samples collected for testing	3.4.2.1
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	3.4.2.2
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	3.4.2.3
	20	Any adverse events from performing the index tests or the reference standard.	3.4.2.4
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	3.4.3.1
	22	How indeterminate results, missing data and outliers of the index tests were handled.	3.4.3.2
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	3.4.3.3
	24	Estimates of test reproducibility, if done.	3.4.3.4
DISCUSSION	25	Discuss the clinical applicability of the study findings.	3.5

Table reproduced from Bossuyt *et al* (Bossuyt et al. 2003)

3.2 Title /Abstract/Keywords

Title

An evaluation of the diagnostic accuracy of the 3rd generation RIDASCREEN norovirus ELISA and the RIDAQUICK immunochromatographic norovirus test including a comparison of both to the second generation IDEIA norovirus ELISA.

Abstract

The laboratory diagnosis of norovirus infection allows the appropriate management of norovirus outbreaks and norovirus infected patients. It also allows the undertaking of studies into the clinical and molecular epidemiology of norovirus. Two new commercial diagnostic tests have become available for the diagnosis of norovirus infection. These are the RIDASCREEN ELISA and RIDAQUICK immunochromatographic test. These tests were evaluated on a collection of samples from children with acute gastroenteritis in Brazil, 96 with norovirus and 116 without norovirus in their faeces by PCR. Of the 96 norovirus positive samples, 66 were from children hospitalised with gastroenteritis and 30 from community based cases of gastroenteritis. The RIDASCREEN ELISA was found to have a sensitivity of 90% at 80% specificity, in children admitted to hospital with gastroenteritis. This makes it suitable for the testing of samples for norovirus in hospital based epidemiological studies prior to molecular analysis. The RIDASCREEN ELISA and RIDAQUICK immunochromatographic tests, compared to a PCR test, are 70 and 79%

sensitive respectively for the detection of norovirus in children hospitalised with gastroenteritis, both with 100% specificity.

Keywords

Norovirus; ELISA; Chromogenic; Test; Sensitivity; Specificity

3.2.1 Aims

1- To estimate the diagnostic accuracy of the IDEIA norovirus 2nd generation ELISA (Oxoid), with PCR based norovirus detection as the gold standard test.
2- To estimate the diagnostic accuracy of the RIDASCREEN 3rd generation norovirus ELISA (R-Biopharm), with PCR based norovirus testing as the gold standard test.
3- To estimate the diagnostic accuracy of the RIDAQUICK norovirus ICG test (R-Biopharm), with PCR based norovirus testing as the gold standard test.
4- To compare the diagnostic accuracy of the RIDASCREEN and RIDAQUICK tests to the ELISA test that has the best sensitivity in published evaluations, the IDEIA norovirus ELISA (Oxoid).
5- To consider the clinical utility and epidemiological survey utility of the RIDASCREEN 3rd generation norovirus ELISA, IDEIA ELISA and the RIDAQUICK norovirus immunochromatographic test based on their analytical and diagnostic accuracy.

3.3 METHODS

3.3.1 PARTICIPANTS

3.3.1.1 Study population & 3.3.1.2 Participant recruitment & 3.3.1.3 Participant sampling

Investigators at the Liverpool School of Tropical Medicine, UK (Dr L Cuevas) and the Federal University of Sergipe, Brazil (Dr R Gurgel) undertook a study of viral gastroenteritis in Brazilian children. The aim of this study was principally to monitor the epidemiology of rotavirus infection following the introduction of rotavirus vaccination. The study ran from October 2006 to September 2009, although only samples from October 2006 to December 2007 were used for the study presented in this chapter. This led to faecal samples being collected from two paediatric cohorts. The cohorts were separated into children presenting to hospital and children in the community. The hospital group comprised children with diarrhoea of <7 days duration, samples were collected consecutively over 4 days each week. The hospitals attended were João Alves Hospital and Hospital Municipal da Zona Norte, Aracaju, Brazil. Data was collected prospectively by medical staff at the time of hospital admission. The community based cohort consisted of children <5 years old in Santa Maria District, Aracaju, an area with low cost houses and some additional un-planned houses. Samples were collected by parents and taken to a local health centre. Data was collected prospectively by community based nursing staff visiting the homes of symptomatic children on an ongoing basis.

Inclusion criteria: Meeting the description of the community or hospital criteria as described above.

Exclusion criteria: Children were excluded if they were non-permanent residents, homeless, with major congenital anomalies or without a legal guardian or parent.

Ethics: Ethical approval was granted by the Liverpool School of Tropical Medicine Research Ethics Committee, the Ethical Committee of the Federal University of Sergipe and Brazil´s National Ethics Committee (CONEP) (see Appendix).

3.3.1.4 Data collection

Samples and data collected from study participants was collected prospectively.

3.3.2 Test methods

Processing pathway of faecal samples

After samples were collected they were stored at -70^{0}C in Brazil. They were sent to the UK on ice where they were stored at -70^{0}C. Samples were defrosted to make 10% faecal suspensions in PBS which were stored at -70^{0}C being defrosted once before testing by norovirus PCR. A selection of the original samples, based on their PCR result, were then defrosted and made into suspensions in the recommended diluents, to allow ELISA/ICG testing to be performed. ELISA/ICG testing was carried out on the same day as the ELISA/ICG suspensions were made.

3.3.2.1 TESTS UNDER EVALUATION

3.3.2.1.1 IDEIA norovirus ELISA (Oxoid, Thermo Fisher Scientific, Basingstoke, UK).

IDEIA norovirus ELISA: Principle of test

Microwells in a microtitre plate are coated with antibodies against norovirus antigens. A faecal suspension is mixed with monoclonal and polyclonal anti-norovirus antibodies which are conjugated to horseradish peroxidase. These are added to the microwells and sandwich the viral antigens to the surface of the microwells during an incubation step. After non sandwiched sample/reagents are washed out from the well, a chromogen is added to the microwells and incubated. The reaction is then stopped and the change in colour is detected by a spectrometer and a result derived based on the value of the negative control.

IDEIA norovirus ELISA: Test procedure

Preparation: All reagents and the microwell plate were brought to room temperature (20–25°C) before use, following storage at 2-8^{0}C. The microwell strips were not removed from their aluminium storage bag until they reached room temperature. The reagents were thoroughly mixed immediately before use. Wash buffer was prepared to a working concentration. Samples were prepared by diluting 100μl of liquid stool or 50-100mg of solid stool into 1ml of sample dilution buffer. Samples were homogenised by mixing with a vortex, then left to stand for 10 minutes and the supernatant used in the test described below.

The test: Samples were pipetted into the microwells as well as a negative control (sample dilution buffer) and a positive control (100μl). 100μl of peroxidase labeled monoclonal and polyclonal antibodies were added to each well and mixed by gentle tapping of the side of the microtitre plate. The microtitre plates were then left at 20-25^{0}C for 1 hour. The wells were then washed by emptying the wells with the plate being knocked onto absorbent paper to remove residual moisture. Five washes were then carried out for each microwell. This involved adding 350μl of wash buffer to each well followed by knocking plates onto absorbent paper to remove residual moisture. After the wash 100μl of chromogen was added to each well and incubation at 20-25^{0}C for 30 minutes followed. After this, 100μl of stop reagent was added to each well. The samples absorbance was then read with a microtitre plate reader (FLUOstar Omega, BMG LABTECH, Aylesbury, UK) at 450nm with blank correction.

Quality control: Positive and negative controls were used with each batch of samples. The test results were accepted if the optical density (O.D.) for the negative control was less than 0.15 at 450nm and the positive control is greater than 0.5 at 450nm. The IDEIA norovirus kit lot numbers used in this study were all lot no 773832.

3.3.2.1.2 RIDASCREEN norovirus ELISA (R-Biopharm, Darmstadt, Germany).

RIDASCREEN norovirus ELISA: Principle of test

Microwells in a microtitre plate are coated with antibodies against norovirus antigens. A faecal suspension is mixed with a biotinylated monoclonal anti-norovirus antibody. These are added to the microwells and sandwich the viral antigens to the surface of the microwells during an incubation step. After non sandwiched sample/reagents are washed out from the well a conjugate, streptavidin peroxidase, is added to the microwell. Incubation, to allow binding of the streptavidin peroxidase to the biotinylated monoclonal anti-norovirus antibodies follows. Unattached streptavidin peroxidase conjugate is removed during a further washing phase. A chromogen is then added which the streptavidin peroxidase acts upon. The reaction is then stopped after a further incubation using a stop solution. The change in colour is then detected by a spectrometer and a result derived based on the value of the negative control.

RIDASCREEN norovirus ELISA: Principle of test

Preparation: all reagents and the microwell plate were brought to room temperature (20–25°C) before use, following storage at 2-8^{0}C. The microwell strips were not removed from their aluminium storage bag until they reached room temperature. The reagents were thoroughly mixed immediately before use. Wash buffer was prepared to a working concentration. Samples were prepared by diluting 100μl of liquid stool or 50-100mg of solid stool into 1ml of sample dilution buffer. Samples were homogenised by mixing with a vortex. Samples were left to stand for 10 minutes and the supernatant used in the test described below.

The test: Samples, a negative control (sample dilution buffer) and a positive control (100μl) were pipetted into microwells. 100μl of biotin-conjugated antibody was added and the resulting mixture mixed by gentle tapping of the side of the microtitre plate. Incubation at room temperature (20 – 25 °C) for 60 minutes followed before the microwells were washed. Firstly, the contents of the wells were emptied with the plates being knocked onto absorbent paper to remove residual moisture. Five washes were then carried out for each microwell involving addition of 300μl of wash buffer. After the wash, 100μl of the streptavidin-peroxidase conjugate was added to the wells and incubated at room temperature (20 – 25 °C) for 30 minutes. Five washes were again completed followed by 100μl of substrate being added to each well. The plate was then incubated at room temperature (20 - 25 °C) for 15 minutes in the dark. After this, 50μl of stop reagent was added to each well. The samples absorbance was then read with a FLUOstar Omega microtitre plate reader read at 450nm, with blank correction.

Quality control: Positive and negative controls were used with each batch of samples. The test results were accepted if the O.D. for the negative control was less than 0.2 at 450nm and the positive control greater than 0.5 at 450nm. The RIDASCREEN norovirus lot numbers used in this study were all lot no. 02069.

3.3.2.1. 3 RIDAQUICK norovirus immunochromatographic test (R-Biopharm, Darmstadt, Germany).

RIDAQUICK norovirus immunochromatographic test: Principle of test

A membrane provides the surface for the test to take place on, as opposed to a microwell in an ELISA test. The membrane has a test section and control section, see Figure 3.1. The test section has anti-norovirus antibodies and the control section contains anti-mouse IgG antibodies. A sample supernatant is mixed with biotinylated antibodies against norovirus antigens. This mixture is then added to the membrane which, if it contains norovirus antigens, may bind to the immobilised anti-norovirus antibodies. The biotinylated antibodies, present in excess, with no antigens bound will bind to the control line. Streptavidin with horseradish peroxidise attached is then added which will bind to the biotinylated antibodies. The non-bonded peroxidase is removed by adding Wash buffer to the reaction window. A chromogen is then added to detect bound peroxidase as an indicator of the presence of norovirus antigen in the sample.

RIDAQUICK norovirus immunochromatographic test: Test procedure

Preparation: All reagents, and the microwell plates, were brought to room temperature (20–25 °C) before use, following storage at 2-8^{0}C. The RIDAQUICK test cartridges were not removed from their aluminium storage bag until they reached room temperature. The reagents were thoroughly mixed immediately before use. Samples were prepared by diluting 100μl of liquid stool or 50-100mg of solid stool into 1ml of sample dilution buffer. Samples were homogenised by mixing with a vortex and left to stand for 2 minutes, with the supernatant used in the test as described below.

The test: The test cassette was laid on a flat surface. 250μl of stool suspension supernatant was mixed with 6 drops of biotinylated antibodies against norovirus antigens. The mixture was added to the sample test well, horizontal to T label (Figure 4.1), at 45^0 with the point of the pipette used aimed towards the reaction window. After 10 minutes streptavidin with horseradish peroxidise was added to the reaction window. After 1 minute, 10 drops of wash buffer were added followed by 6 drops of the chromogen. The reaction result was read within 3 minutes.

Quality control: A blue line was required to be visible in the control section (C) of the test for the test result to be valid. The blue control band confirms that the sample and the reagents have been added correctly, the reagents were active when the test was carried out and the sample has migrated properly through the test membrane. A colourless background in the result zone (reaction window) acts as an internal negative control. The RIDAQUICK norovirus test lot numbers used were lot no. 02408, 03049, 03448 and 05139.

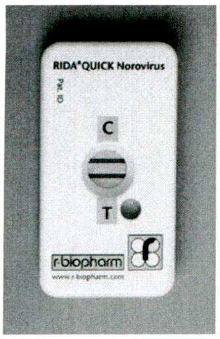


Figure 3.1: The norovirus RIDAQUICK ICG test. Visual reading of the blue control (C) and test (T) line gives a result.

3.3.2.2 Gold standard tests

Polymerase chain reaction (PCR) tests. Real time and qualitative reverse transcriptase PCR (RT-PCR) were combined to form the gold standard test, they are now described.

Principle of test: Qualitative RT-PCR consists of the following stages. RNA extraction: RNA is extracted from the clinical samples to provide an RNA template for reverse transcription. RNA is reverse transcribed using a reverse transcription enzyme providing cDNA for the PCR step. PCR: cDNA is amplified using a DNA polymerase reaction to provide a product of a specific size. The size of the product is dependent upon the primers used. Agarose gel electrophoresis: The product produced by the PCR is stained with ethidium bromide and separated on an agarose gel. The product is visualised and the size of the product is confirmed as being compatible with that expected by comparison to a positive control and a molecular weight ladder. In real time RT-PCR a probe with the potential to fluoresce is included in the PCR reaction mixture. This binds specifically to cDNA until cDNA is replicated by DNA polymerase. DNA polymerase removes the probe which will then fluoresce. The fluorescence detected is proportional to the specific replication of the norovirus cDNA.

3.3.2.2.1 Real time RT-PCR

Real time RT-PCR: Test procedure

Preparation: see section 2.3.2.1

The test: RNA extraction and reverse transcription: see section 2.3.2.1.

Real time PCR: the PCR mixture was prepared containing:

Sensimix (Quantace, London, UK)	12.5μl
HPLC water	6.375μl
10μM primers	1μl
(Primers = COG1F and COG1R (GI), or COG2F and COG2R (GII) (Operon, Leeds, UK))	
20μM Probe	0.125μl
(Probe = Ring 1AB/TP (GI) or Ring 2 AB/TB (GII) (Operon))	

cDNA 5μL was added to this mix before PCR was carried out under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C (denaturation) for 15 seconds and 56°C (annealing) for 1 minute. GI PCR was undertaken on the Light cycler (Roche 480) and the GII on the Rotagene 6200-HRM (QIAGEN). Different machines were used to allow more rapid processing of samples.

All primers and probes have been previously published. They were purchased at 50nmole and HPLC purified, the sequences of which were (Kageyama et al. 2003);

Primers:

COG1-F CGYTGGATGCGNTTYCATGA (forward primer)

COG1-R CTTAGACGCCATCATCATTYAC (reverse primer)

COG2-F CARGARBCNATGTTYAGRTGGATGAG (forward primer)

COG2-R TCGACGCCATCTTCATTCACA (reverse primer)

Probes:

Ring1ABFAM [6-FAM]AGATYGCGRTCYCCTGTCCA[BHQ1a-6FAM]

Ring2TPFAM [6-FAM]TGGGAGGGCGATCGCAATCT[BHQ1a-6FAM]

Quality control: Test results were verified by the inclusion of positive and negative controls in each batch of samples.

3.3.2.2.2 **Qualitative RT-PCR:** See section 2.3.3.1.

3.3.2.3 ADDITIONAL TESTS: METHODS

3.3.2.3.1 **DNA purification:** See 2.3.3.2

3.3.2.3.2 **Sequencing of PCR products:** See 2.3.3.3

3.3.2.3.3 **Processing sequence data:** See 2.3.3.4

3.3.2.4 Categorisation of test results

Categorisation of results by the gold standard.

Positive results by PCR

The gold standard used in this study is defined by two PCR based tests carried out in series. Firstly, real time RT-PCR was carried out. Samples positive by this test were confirmed by qualitative RT-PCR. Both tests were required to be positive for the sample to be considered as positive for norovirus. Given excretion of norovirus in faeces when clinical symptoms are present, occurs at high levels, both assays should detect infection causing clinical gastroenteritis.

Real time RT-PCR. A fluorescence level of over for >1 (Light cycler 480, Roche) for GI testing and >10 (Rotagene 6200-HRM, QIAGEN) for GII testing was accepted as a positive RT-PCR result. In our experience real time RT-PCR can very occasionally give products which are not confirmed by qualitative PCR e.g. a product not of this appropriate size is produced by qualitative PCR.

Qualitative PCR. Qualitative PCR produces a product of a specific size which can be visualised when the product is stained and run on an agarose gel. It is possible that non-specific products are formed which produce a product of the same size as a norovirus product (Zintz et al. 2005).

Given both real time PCR and qualitative PCR can give false positive results, both real time RT-PCR and qualitative PCR were combined to improve the specificity of the reference standard.

Negative results by the gold standard

A sample negative by real RT-PCR is considered as negative.

Equivocal results by the gold standard.

Results were only categorised as positive or negative, no equivocal category was used.

Categorisation of results by the evaluated tests

IDEIA norovirus ELISA

Categorising a samples test result was carried out according to the manufacturer interpretation of sample absorbance of 450nm wavelength light. Firstly cut off values were calculated. The cut-off is equal to the O.D. for the negative control + 0.1. A result is positive if the results are >0.01 above the cut off. A result is negative if the results is <0.01 below the cut off. An equivocal range exists and is ± 0.01 of the cut-off. Equivocal results require repeating.

RIDASCREEN norovirus ELISA

Categorising a samples test result was undertaken according to the manufacturer's interpretation of sample absorbance of 450nm wavelength light. Firstly, cut off values were calculated. The cut-off is equal to the O.D. for the negative control + 0.15. A result is positive if the result is >10% above the cut off. A result is negative if the results is <10% below the cut off. An equivocal range exists and is ± 10 % of the cut-off. Equivocal results require repeating.

RIDAQUICK norovirus ICG test

Categorising a samples test result was undertaken according to the manufacturer instructions. A positive result had any intensity blue line visible on the test strip of the test. A negative result had no blue line visible on the test strip.

3.3.2.5 Competencies of staff completing laboratory work

The gold standards of real time RT-PCR and qualitative PCR were conducted by three people (A. Booth, W. Dove, A. Kirby; Liverpool University) with experience in completing PCR for viral agents of gastroenteritis. The ELISA and ICG tests were undertaken by a single person

(A. Kirby) with experience of undertaking immunological tests. ELISA results were obtained by an automatic plate reader. Where visual readings were carried out for the ICG test, a second person assessed the results. The second reader of the ICG test was blind, blinding of the ELISA test was not relevant as the result was read by a plate reader. The result of the PCR test was available to the person completing the ELISA tests, but no clinical information was available to the persons completing the PCR testing.

3.3.2.6 Potential for result bias

PCR: No clinical information was available to allow bias to enter the PCR results.

ELISA and ICG: The PCR results were available when completing ELISA and ICG testing and had the potential to bias the ELISA and ICG results. The ELISA results were read by a plate reader removing opportunity for bias. ICG tests were read in duplicate, one person blinded to the PCR result with no disagreement occurring.

3.3.3 Statistical methods

Statistical support was provided by Brian Faragher, Liverpool School of Tropical Medicine, Liverpool University.

3.3.3.1 Sample size calculations

The sample sizes were calculated using the computer program, Epi-info (Version 3.5.1). Sample sizes were calculated with reference to the aims of the study.

1- To estimate the diagnostic accuracy of the IDEIA norovirus ELISA (Oxoid) with PCR based norovirus testing as the reference standard.

- Sample size to estimate the sensitivity of the IDEIA norovirus test, given:

    - The population size was 728. This was the size of the cohort in this study.
    - The test is 59% sensitive (Table 3.01).
    - A lower bound to the tests sensitivity of 49%.
    - The estimate should have a 95% C.I.

This gives a sample size of 82 to estimate the sensitivity of the IDEIA norovirus ELISA.

- Sample size calculation to estimate the specificity of the IDEIA norovirus test given:

    - The population size was 728. This was the size of the cohort in this study.
    - The test is 94% specific (Table 3.01).
    - A lower bound to the tests specificity of 90%.
    - The estimate should have a 95% C.I.

This gives a sample size of 114 to estimate the specificity of the IDEIA norovirus ELISA.

2- To estimate the diagnostic accuracy of the RIDASCREEN 3rd generation norovirus ELISA, R-BIOPHARM, with PCR based norovirus testing as the gold standard.

- Sample size to estimate the sensitivity of the RIDASCREEN norovirus test, given:
    - The population size was 728. This was the size of the cohort in this study.
    - The test is 83% sensitive, as reported by the manufacturer.
    - A lower bound to the tests sensitivity of >75%.
    - The estimate should have a 95% C.I.

This gives a sample size of 96 to estimate the sensitivity of the RIDASCREEN norovirus test

- Sample size calculation to estimate the specificity of the RIDASCREEN norovirus test given:
    - The population size was 728. This was the size of the cohort in this study.
    - The test is 90% specific, a conservative estimate given the claimed 100% specificity, reported by the manufacturer.
    - A lower bound to the tests specificity of 85%.
    - The estimate should have a 95% C.I.

This gives a sample size of 116 to investigate the specificity of the RIDASCREEN norovirus test.

3- To estimate the diagnostic accuracy of the RIDAQUICK norovirus ICG test, R-BIOPHARM, with PCR based norovirus testing as the gold standard.

- Sample size to estimate the sensitivity of the RIDAQUICK norovirus test, given:
    - The population size was 728. This was the size of the cohort in this study.
    - The test is 83% sensitive, as reported by the manufacturer.
    - A lower bound to the tests sensitivity of > 75%.
    - The estimate should have a 95% C.I.

This gives a sample size of 96 to estimate the sensitivity of the RIDAQUICK norovirus test.

- Sample size calculation to estimate the specificity of the RIDAQUICK norovirus test given:
    - The population size was 728. This was the size of the cohort in this study.
    - The test is 95% specific, a conservative estimate given the claimed 100% specificity.
    - A lower bound to the tests specificity of 85%
    - The estimate should have a 95% C.I.

This gives a sample size of 18 to estimate the specificity of the RIDAQUICK norovirus test.

4- To compare the diagnostic accuracy of the RIDASCREEN and RIDAQUICK tests to the ELISA test that had the best sensitivity in published evaluations, the IDEIA norovirus ELISA.

- Sample size calculation to compare the sensitivity of the RIDASCREEN and RIDAQUICK norovirus tests to the IDEIA ELISA given:
  - The sensitivity of the RIDASCREEN and RIDAQUICK norovirus tests are 83% as reported by the manufacturer.
  - The sensitivity of the IDEIA norovirus test is 59% (Table 3.01).
  - A 95% C.I. and 80% power.

This gives a sample size of 81 to compare the sensitivity of the RIDASCREEN and RIDAQUICK IDEIA norovirus tests.

Sample size calculation to compare the specificity of the RIDASCREEN and RIDAQUICK IDEIA norovirus tests. This was not carried out as both report specificities of 100%.

3.3.3.2 Methods for interpretation of results

Reporting of diagnostic accuracy. Reporting of results is carried out by a cross tabulation table as shown below (Table 3.07).

Table 3.07: Cross tabulation for presenting study results.

		PCR	
Test under evaluation		+	-
	+	True positives	False positives
	-	False negatives	True negatives

True positive (TP): A sample testing positive by the test under evaluation and the reference standard.

False positive (FP): A sample testing positive by the test under evaluation and negative by the reference standard.

False negative (FN): A sample testing negative by the test under evaluation and positive by the reference standard.

True negative (TN): A sample testing negative by the test under evaluation and the reference standard.

The reference standard in this study, norovirus PCR, is used to define the presence of norovirus disease.

The following characteristics of the tests/statistical tests will be presented

1. **Sensitivity**= True positives / True positives + False negatives
2. **Specificity**= True negatives / True negative + False Positives

These values will be presented with 95% confidence intervals.

3. **Negative predictive value (NPV)**, based on the prevalence of norovirus in the population.

   NPV= True negatives/True negatives + False negatives

4. **Positive predictive value (PPV)**, based on the prevalence of norovirus in the population.

   PPV=True positives/True positives + False positives

Prevalence rates, as defined by PCR, are: overall 13.23%, hospital; 66/393= 16.8% and community=30/335 = 9%.

5. **Positive predictive value adjusted for detection of asymptomatic shedding**

Positive predictive values will be calculated for PCR and the RIDASCREEN/ RIDAQUICK ELISA for the hospital cohort, given this cohort is most likely to undergo diagnostic testing, taking account of clinical false positives. A clinical false positive is where the diagnostic test detects a pathogen (norovirus) but that pathogen is not causing clinical disease. The clinical false positive prevalence is estimated below, for PCR and ELISA testing, and is considered equal to the detection of asymptomatic shedding.

Calculating the detection of asymptomatic shedding by PCR/ELISA methods.

To calculate the detection of asymptomatic shedding of norovirus at any time point in a random sample of patients the following information is needed:

- The percentage of the population susceptible to norovirus infection
- The frequency of norovirus infection within the susceptible population
- The duration of asymptomatic shedding

Estimating the percentage of the population susceptible to norovirus infection

- ➢ 80% of the population are susceptible to norovirus infection (Marionneau et al. 2002)

Estimating the frequency of infection

- Immunity to norovirus infection has been estimated as lasting 6-12 months (Midpoint = 9 months)(Dolin et al. 1972; Wyatt et al. 1974; Parrino et al. 1977).
- Infection in children is assumed to occur when immunity is absent.
  - ➢ Infection can be estimated at occurring every 9 months

Estimating the duration of asymptomatic shedding

- ➢ Asymptomatic shedding has been estimated as lasting for an average of 28 days as detected by norovirus PCR (Murata et al. 2007; Atmar et al. 2008; Kirkwood and Streitberg 2008).
- ➢ Asymptomatic shedding has been estimated as lasting for an average of 7 days after infection as detected by norovirus ELISA (Graham et al. 1994; Okhuysen et al. 1995; Atmar and Estes 2001).

Calculation the prevalence of asymptomatic shedding of norovirus by:

PCR: The proportion of time between norovirus infections is 9 months, and PCR will detect shedding for 28 days within this period. This will occur in 80% of the population. Therefore asymptomatic excretion of norovirus will occur for 1 month in 80% of the population every 9 months ((28days /270 days) x0.8) = 8%

ELISA: The proportion of time between norovirus infections is 9 months, and ELISA will detect shedding for 7 days within this period. This will occur in 80% of the population. Therefore asymptomatic excretion of norovirus will occur for 7 days in 80% of the population every 9 months ((7 days /270 days) x0.8) = 2%

The decision to use these estimates of clinical false positives to calculate norovirus tests positive predictive values is supported by studies which have shown an approximately 10% rate of asymptomatic shedding (Farkas et al. 2000; Monica et al. 2007; Reither et al. 2007). It is though evident there is a wide range of shedding seen studies of asymptomatic shedding; from 1-30% (O'Ryan et al. 2000; Castilho et al. 2006). Further support for the use of these estimates is given by a recent Brazilian study which showed an asymptomatic shedding rate as detected by PCR of 12%, a figure comparable to the estimate above.

6. **McNemar`s test.** To compare the diagnostic accuracy of the RIDASCREEN and RIDAQUICK tests to the ELISA test that had the best sensitivity in published evaluations, the IDEIA ELISA, the McNemar`s test will be used. This will calculate if there is a statistical difference in the results of the different norovirus tests as the data is categorical, summarised as a percentage and paired.

7. **Receiver operating characteristic curves.** An evaluation of the ELISAs test cut off will be made by producing receiver operating characteristic (ROC) curves. ROC curves show graphically sensitivity against (1 - specificity). This allows consideration of what is an appropriate cut off point; different cut off points will give different sensitivity and specificity characteristics to the ELISAs. The ELISA values will be normalised between the different ELISA test runs by dividing a samples optical density by that of its respective negative control. E.g. an O.D. value of 0.64 where the negative control had an O.D of 0.32 would have a normalised value of 2 (0.64/0.32). Sensitivity and specificity values will be calculated at separations of the normalised values of 0.1 e.g. 0.1, 0.2, 0.3. The ROC analysis will

specifically analyse the sensitivity of the ELISAs at 80% test specificity. This is a specificity that may be acceptable if using the ELISA to screen a sample collection prior to completing PCR.

3.3.3.3 Test reproducibility

Test reproducibility was assessed for the ELISA tests in terms of run to run variability with new samples prepared. Run to run variability was measured by examining the variation in O.D. values. This variation was examined in three categories, each containing eight samples. The categories were: ELISA and PCR positive samples, ELISA and PCR negative samples and samples with discordant ELISA and PCR samples or variation in the ELISA test result being observed during the evaluation e.g. equivocal results becoming positive. The reproducibility is expressed as a coefficient of variation (CV); this is calculated by dividing the standard deviation by the mean optical density.

3.3.3.4 Impact of clinical features on test outcomes.

The usefulness of immunological testing for norovirus is likely to be affected by a number of clinical characteristics. In the case of norovirus these are principally the time since diarrhoea onset at the time of testing and the clinical severity of the disease. Therefore, an analysis of the impact of duration of diarrhoea at the time the sample was tested and the clinical severity of the disease on the RIDASCREEN/RIDAQUICK results within the hospital cohort was carried out.

3.4 RESULTS

3.4.1 PARTICIPANTS

3.4.1.1 Timing of sample collection and study

The samples were collected between October 2006 and December 2007.

Testing was carried out in May-June 2009.

3.4.1.2 Clinical and demographic characteristics

Hospital cohort

Age: 0 months old to 12 years old. Mean age (S.D): 21 (28) months.

Sex: Male 57%, Female 43%.

Mean (SD) duration of diarrhoea at presentation of 4.21 (3.1) days (range 0 – 15).

Community cohort.

Age: 0 months to 5 years old. Mean age (S.D) 18 (6) months

Sex: 50% Male, 50% Female.

Mean (SD) duration of diarrhoea of 4.27 (2.6) days (range 1-35).

Duration of diarrhoea in the community cohort was recorded at a separate time from presentation so is not directly comparable to duration of diarrhoea in the hospital cohort.

Duration was assessed by a home visit programme by community based nursing staff which may or may not have been related to the diarrhoeal episode.

3.4.1.3 Outcomes of included and excluded patients

All patients who satisfied the inclusion criteria underwent real time RT-PCR for the detection of norovirus. All patients with positive RT-PCR and qualitative PCR results with sufficient sample underwent ELISA testing. A collection of samples from patients who tested negative by real time RT-PCR (systematically selected) were screened by ELISA.

3.4.2 TEST RESULTS

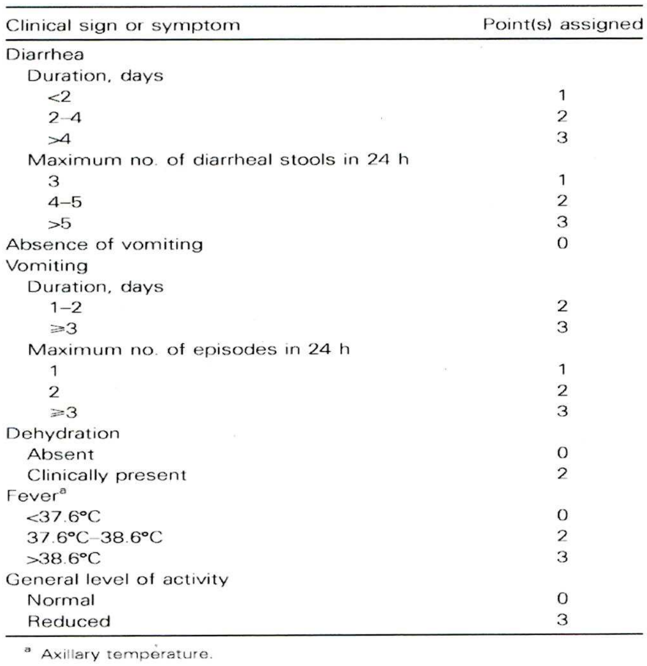
3.4.2.1 Time interval between samples collected for testing

A single sample was collected at a single time point to investigate a patient's gastroenteritis. Samples used for the study underwent both the reference standard test and the immunological norovirus tests being evaluated.

3.4.2.2 Disease severity in participants

The severity of illness was assessed according to the modified Vesikari score (Table 3.08) (Nakagomi et al. 2005). This is used to assess the severity of rotavirus gastroenteritis but is used in this study as no clinical severity score exists for norovirus and the clinical features of the two conditions are similar. In the community cohort the modified Vesikari score was 8.8 and 10.05 in the hospital cohort.

Table 3.08: The 20 point modified Vesikari Severity Scoring System used to evaluate the severity of rotavirus gastroenteritis (Nakagomi et al. 2005).



Clinical sign or symptom	Point(s) assigned
Diarrhea	
Duration, days	
<2	1
2–4	2
>4	3
Maximum no. of diarrheal stools in 24 h	
3	1
4–5	2
>5	3
Absence of vomiting	0
Vomiting	
Duration, days	
1–2	2
⩾3	3
Maximum no. of episodes in 24 h	
1	1
2	2
⩾3	3
Dehydration	
Absent	0
Clinically present	2
Fever[a]	
<37.6°C	0
37.6°C–38.6°C	2
>38.6°C	3
General level of activity	
Normal	0
Reduced	3

[a] Axillary temperature.

Table reproduced from Nakagomi *et al* (Nakagomi et al. 2005)

3.4.2.3 Cross-tabulation of results

Overall (community and hospital cohorts) results

The sensitivities of the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests were 64%, 69% and 49% respectively. Their specificities were all 100%. These values are based on faecal samples collected from the children in both the community and hospital cohorts (Table 3.09).

Table 3.09: Sensitivity and specificity of the RIDASCREEN/RIDAQUICK and IDEIA norovirus tests compared to PCR for the hospital and community cohorts.

	RIDASCREEN	RIDAQUICK	IDEIA
Sensitivity True positives/All positives (%)	61/96 (64%)	66/96 (69%)	47/96 (49%)
Specificity (%) True negatives/All negatives (%)	116/116 (100%)	20/20 (100%)	116/116 (100%)

3.4.2.4 Adverse events from testing

No adverse events from testing were recognised

3.4.3 ESTIMATES

3.4.3.1 Estimates of diagnostic accuracy

The confidence intervals for the RIDASCREEN, RIDAQUICK and IDEIA tests sensitivities were 54-72%, 58-78% and 39-59% respectively. All tests had similarly high positive and negative predictive values (Table 3.10).The RIDASCREEN and RIDAQUICK tests were both more sensitive than the IDEIA ELISA (p= <0.01) (Table 3.11).

Table 3.10: Overview of the sensitivity, specificity, negative and positive predictive values of the RIDASCREEN and IDEIA norovirus ELISA and RIDAQUICK ICG test compared to PCR for the hospital and community cohorts.

Test	Sensitivity	Sensitivity 95% C.I	Specificity	Specificity 95% C.I	PPV[1]	NPV[1]
RIDASCREEN	64%	54-72%	100%	96-100%	100%	95%
RIDAQUICK	69%	58-78%	100%	80-100%	100%	95%
IDEIA	49%	39-59%	100%	96-100%	100%	93%

[1] Predictive values calculated using prevalence's derived from norovirus PCR testing.

Table 3.11: Statistical difference in sensitivity of the RIDASCREEN and RIDAQUICK tests compared to the IDEIA norovirus test. P values calculated using McNemar`s test.

	RIDASCEEN	RIDAQUICK	IDEIA	P value RIDASCREEN vs. IDEIA	P value RIDAQUICK vs. IDEIA
Hospital and community cohort	64 (54-72) %	69 (58-78) %	49 (39-59) %	0.001	0.00002

3.4.3.2 Handling of indeterminate results, missing results and outliers

No results were indeterminate. No data were missing from the results of the norovirus testing

No outliers were recognised. ELISAs were repeated if there was discrepancy between the result of an ELISA and PCR and there was a discrepancy between ELISA results and if the ELISA result was equivocal. If a repeat result was consistent with other results where previously it was discrepant then the repeat result was accepted as that samples result. A sample without a positive result after two tests, e.g. 2 equivocal results by ELISA, would also be called negative. No repeat ICG tests were undertaken. In total, 11/96 RIDASCREEN results were repeated. Three of these samples changed result; two from negative to positive and one from positive to negative. The IDEIA test required 25 tests to be repeated, four of these resulted in the result changing, three from negative to positive and one from equivocal to positive. Within the negative by PCR samples four samples were positive by ELISA, therefore qualitative PCR, as opposed to real time PCR, was carried out on these samples. All four were positive by qualitative PCR and the samples were removed from the evaluation of test specificity.

3.4.3.3 Estimates of variability of diagnostic accuracy between subgroups of participants.

Subgroup analysis of the results in different patient groups is shown below.

Hospital cohort

The sensitivities of the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests were 70%, 79% and 62% respectively. Their specificities were all 100%. These values are based on faecal samples collected from the children in the hospital cohort (Table 3.12).

Table 3.12: Sensitivity and specificity of the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests compared to PCR for the hospital cohort.

	RIDASCREEN	RIDAQUICK	IDEIA
Sensitivity True positives/All positives (%)	46/66 (70%)	52/66 (79%)	41/66 (62%)
Specificity (%) True negatives/All negatives (%)	58/58 (100%)	4/4 (100%)	58/58 (100%)

Community cohort

The sensitivities of the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests were 50%, 47% and 20% respectively. Their specificities were all 100%. These values are based on faecal samples collected from the children in the community cohort (Table 3.13).

Table 3.13: Sensitivity and specificity of the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests compared to PCR for the community cohort.

	RIDASCREEN	RIDAQUICK	IDEIA
Sensitivity True positives/All positives (%)	15/30 (50%)	14/30 (47%)	6/30 (20%)
Specificity (%) True negatives/All negatives (%)	58/58 (100%)	16/16 (100%)	58/58 (100%)

Genogroup analysis

The sensitivity of the RIDASCREEN, RIDAQUICK and IDEIA for detecting genogroup II norovirus was higher than for genogroup I norovirus. Their sensitivities were 67%, 76% and 55% for genogroup II and 13%, 0% and 0% for genogroup I respectively (Table 3.14)

Table 3.14: Sensitivity of the RIDASCREEN, RIDAQUICK AND IDEIA norovirus tests by genogroup, I or II.

	RIDASCREEN		RIDAQUICK		IDEIA	
	GI	GII	GI	GII	GI	GII
Sensitivity	1/8 (13%)	58/86 (67%)	0/8 (0%)	65/86 (76%)	0/8 (0%)	47/86 (55%)

Genogroup II and hospital cohort

The sensitivity of the RIDASCREEN, RIDAQUICK and IDEIA for detecting genogroup II norovirus in the hospital cohort alone was higher than for the hospital and community cohort. Their sensitivities were 75%, 68% and 87% respectively (Table 3.15)

Table 3.15: Sensitivity of the RIDASCREEN and IDEIA norovirus ELISA and RIDAQUICK ICG test within the genogroup II hospital subgroup.

	RIDASCREEN		IDEIA		RIDAQUICK	
	+	-	+	-	+	-
PCR						
+	45	15	41	19	52	8
Sensitivity	75%		68%		87%	

Summary of subgroup analyses

The tests sensitivities varied greatly between subgroups but were found to be highest in the hospital cohort as opposed to the community cohort and genogroup II norovirus as opposed to genogroup I norovirus. The RIDASCREEN and RIDAQUICK tests were more sensitive than or as sensitive as the IDEIA ELISA in all subgroups analysed (3.16).

Table 3.16: Sensitivity, specificity, negative and positive predictive values for the subgroup analysis of the IDEIA and RIDASCREEN norovirus ELISA and the RIDAQUICK ICG test.

Test	Sensitivity	Sensitivity 95% C.I	Specificity	Specificity 95% C.I	PPV[1]	NPV[1]
RIDASCREEN hospital	70	58-80	100%	92-100	1	94
RIDASCREEN community	50	33-67	100%	92-100	1	95
RIDASCREEN G1	12.5	2-47				
RIDASCREEN G2	67	57-76				
RIDASCREEN G2 & Hospital	75	63-84				
RIDAQUICK hospital	79	67-88	100%	51-100	1	96
RIDAQUICK community	47	30-64	100%	81-100	1	95
RIDAQUICK G1	0	0-40				
RIDAQUICK G2	76	65-84				
RIDAQUICK G2 and hospital	87	75-94				
IDEIA Hospital	62	50-73	100%	92-100	1	93
IDEIA Community	20	10-37	100%	92-100	1	93
IDEIA G1	0	0-32				
IDEIA G2	55	44-65				
IDEIA G2 & hospital	68	55-79				

[1] Predictive values calculated using prevalence's derived from norovirus PCR testing.

Norovirus genotype analysis.

The RIDASCREEN, RIDAQUICK and IDEIA tests were 77.6%, 88% and 70.7% sensitive, respectively, at detecting genotype II.4 norovirus. Only small numbers of other genotypes were detected and the tests sensitivity at detecting them was poor (Table 3.17).

Table 3.17: Detection of specific genotypes for the IDEIA and RIDASCREEN norovirus ELISA and the RIDAQUICK ICG test. Two samples with genogroup I and II detected excluded from the analysis.

	RIDASCREEN	RIDAQUICK	IDEIA
GI.3	0/2	0/2	0/2
GI.7	0/1	0/1	0/1
GI.12	0/1	0/1	0/1
GI.14	1/3	0/3	0/3
GII.2	1/4	2/4	1/4
GII.4	45/58 (77.6%)	51/58(88%)	41/58 (70.7%)
GII.6	1/1	1/1	0/1
GII.13	5/7	5/7	1/7
Total	53/77	58/77	43/77

Statistical analysis of variations in sensitivity between the RIDASCREEN, RIDAQUICK and IDEIA norovirus tests.

Where statistical significance ($p=<0.01$) was detected between tests the IDEIA test was found to be less sensitive than the RIDASCREEN and RIDAQUICK tests. This was true of the hospital cohort (RIDAQUICK), community cohort (RIDASCREEN and RIDAQUICK), genogroup II norovirus (RIDASCREEN and RIDAQUICK) (Table 3.18).

Table 3.18: Statistical difference in sensitivity of the RIDASCREEN and RIDAQUICK test compared to the IDEIA norovirus test. P values calculated using McNemar`s test. Analysis conducted on subcategories within the main study.

	RIDASCEEN	RIDAQUICK	IDEIA	P value RIDASCREEN vs. IDEIA	P value RIDAQUICK vs. IDEIA
Hospital	70 (58-80)%	79 (67-88)%	62 (50-73)%	0.18	0.003
Community	50 (33-67)%	47 (30-64)%	20 (10-37)%	0.004	0.008
GI	12 (2-47)%	0 (0-40)%	0 (0-32)%	1.0	1.0
GII	68 (58-77)%	76 (65-84)%	55 (44-65)%	0.007	0.00004
GII and Hospital	75 (63-84)%	87 (75-94)%	68 (55-79)%	0.29	0.003

3.4.3.4 Estimates of predictive values

PPVs of PCR and the RIDASCREEN ELISA based on fixed prevalence's of asymptomatic shedding: The detection of asymptomatic shedding (clinical false positives) by PCR is estimated at 8% and by ELISA at 2%, (see 3.3.3.2). In hospitalised patients the prevalence of norovirus infection by PCR is 16.8% and by ELISA is 70% of that value, 11.76%. Using these estimates the PPV of PCR and the RIDASCREEN ELISA can be calculated (Tables 3.19/3.20). These calculations demonstrate how, based these estimates, PCR for norovirus has a PPV of 52% compared to 83% for the RIDASCREEN test.

Table 3.19: The sensitivity, specificity and PPV of PCR in detecting norovirus in this study against a gold standard which defines PCR as having a clinical false positive rate of 8%.

	Estimated gold standard		
PCR	+	-	PPV
+	8.8	8	8.8/16.8=52%
-	83.2		

Table 3.20: The sensitivity, specificity and PPV of the RIDASCREEN in detecting norovirus in this study against a gold standard which defines ELISA as having a clinical false positive rate of 2%.

	Estimated gold standard		
RIDASCREEN	+	-	PPV
+	9.76	2	9.76/11.76=83%
-	88.24		

3.4.3.5 ROC analysis

The Receiver Operating Characteristic curves shown below (Figure 3.2-3.7) demonstrate that the RIDASCREEN ELISA, in hospitalised patients, had a sensitivity of 90% at an 80% specificity. Reducing the specificity in other situations e.g. IDEIA ELISA and the community cohort, did not usefully improve test specificity.

Figure 3.2: Receiver operating characteristics of the IDEIA ELISA: Community and hospital cohort.

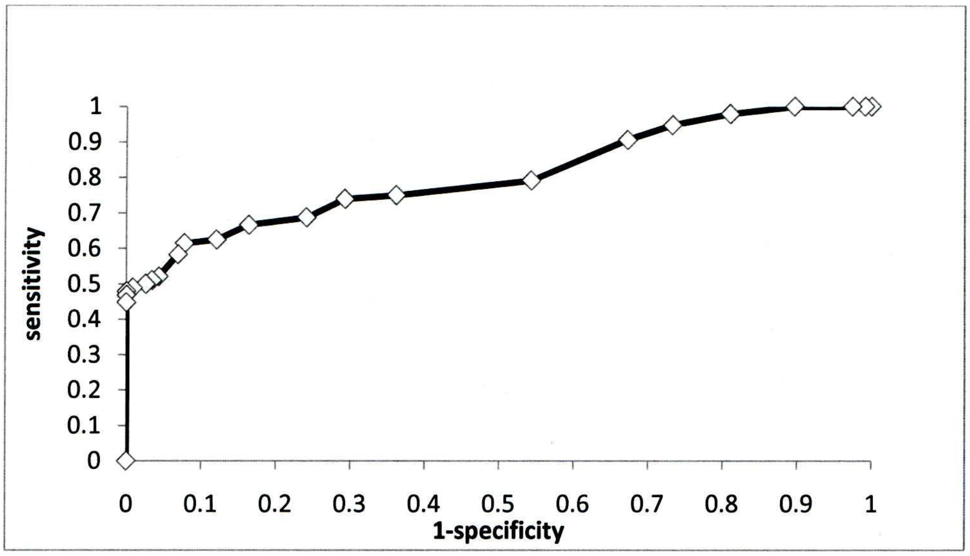


Figure 3.3: Receiver operating characteristics of the IDEIA ELISA: Community cohort.

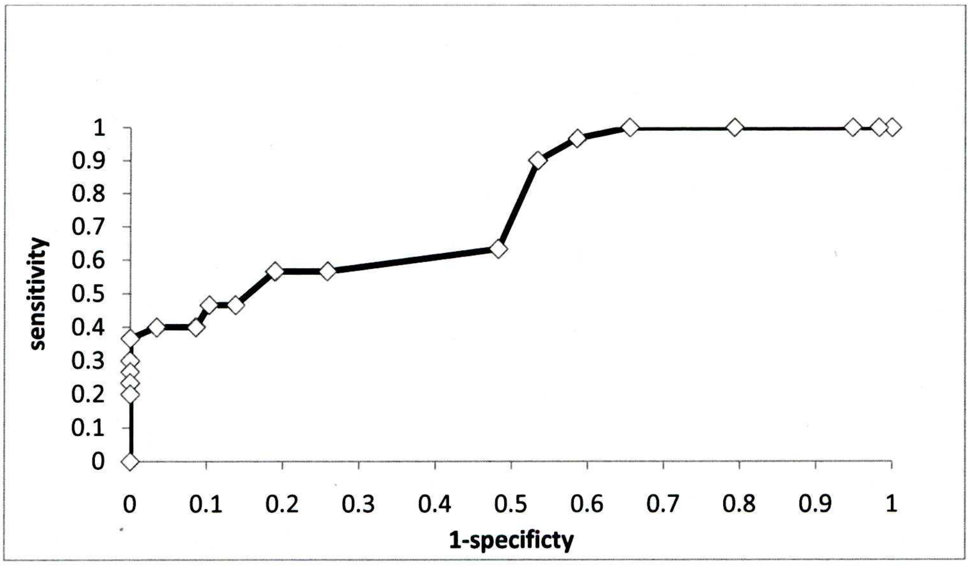


Figure 3.4: Receiver operating characteristics of the IDEIA ELISA: Hospital cohort.

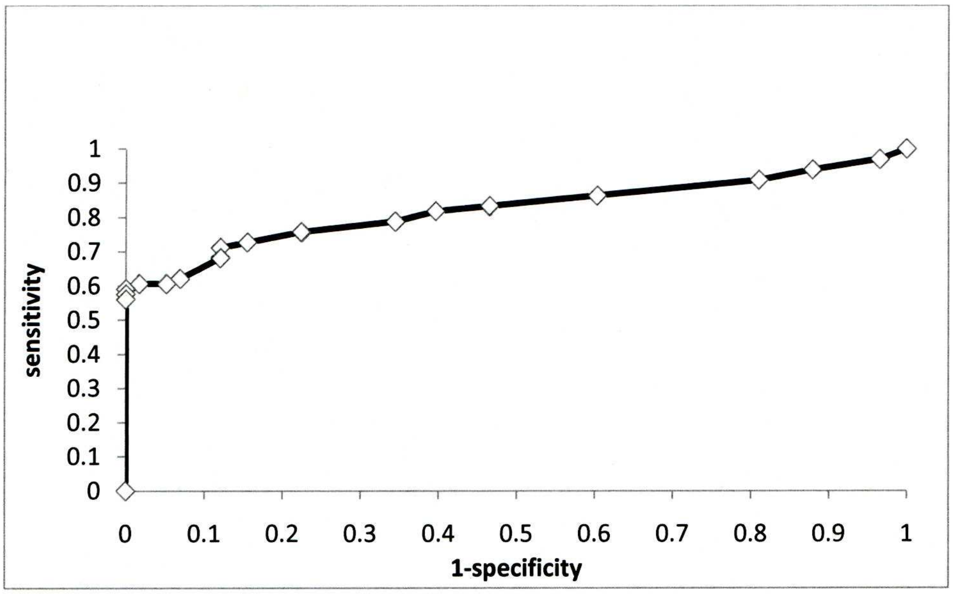


Figure 3.5: Receiver operating characteristics of the RIDASCREEN ELISA: Hospital and community cohort.

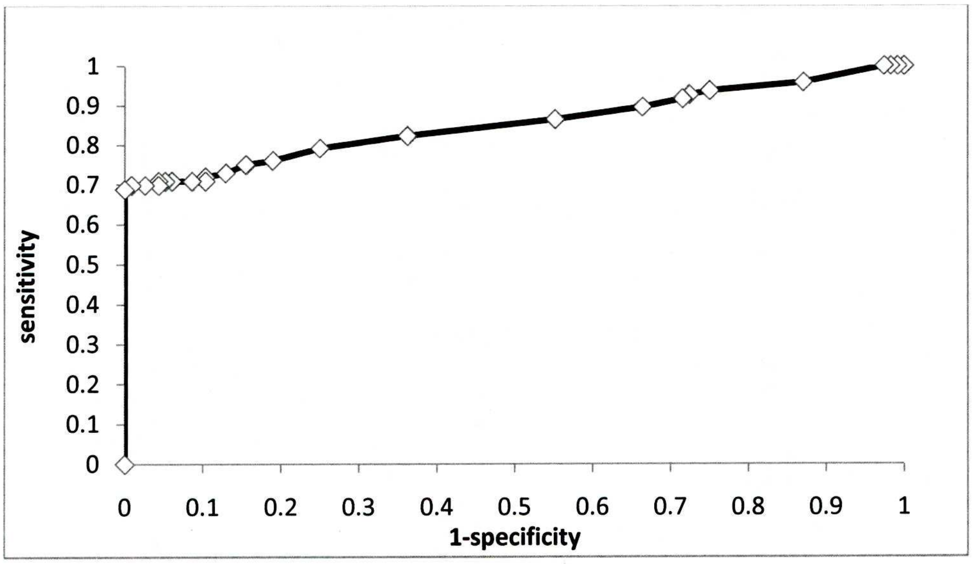


Figure 3.6: Receiver operating characteristics of the RIDASCREEN ELISA: Community cohort.

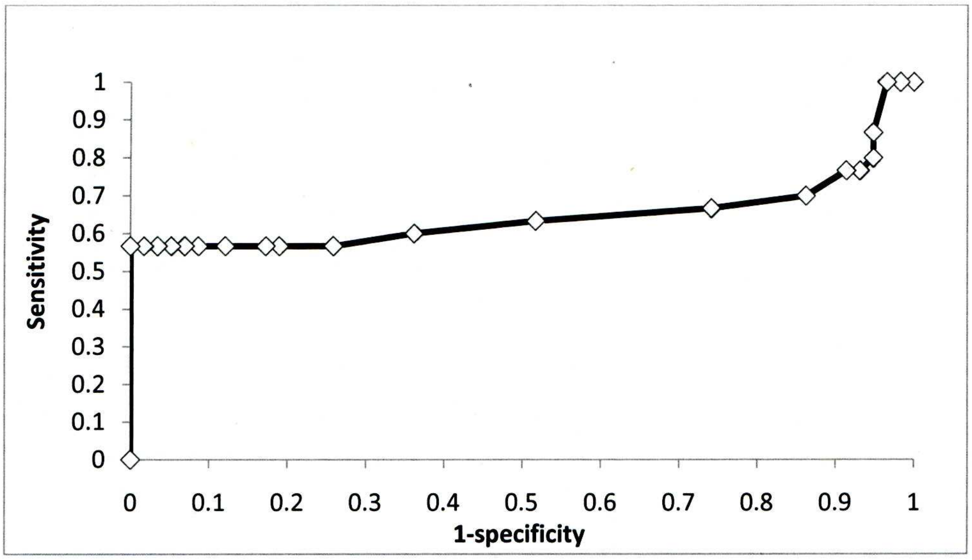
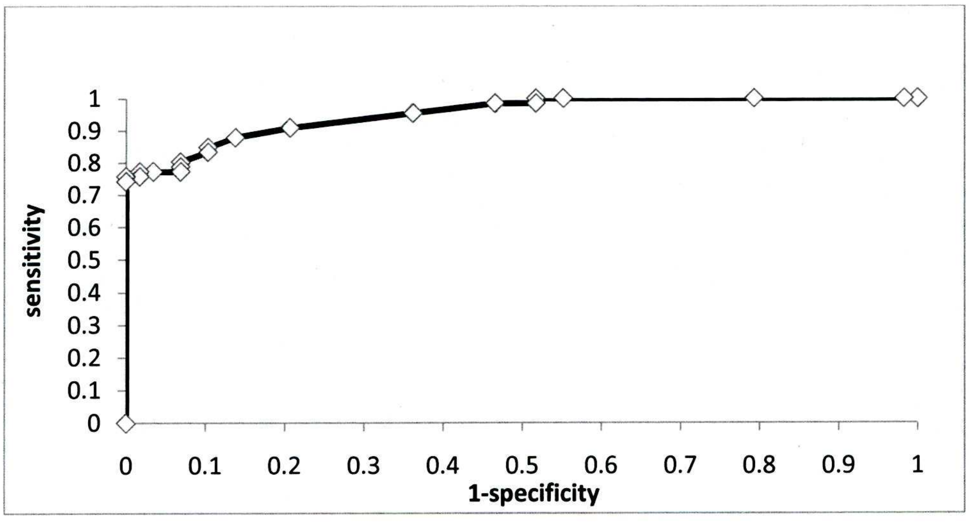


Figure 3.7: Receiver operating characteristics of the RIDASCREEN ELISA: Hospital cohort.



3.4.3.6 Estimates of test reproducibility

Both the RIDASCREEN and IDEIA ELISAs had coefficients of variation of < 25% in samples from the positive and negative groups. The coefficient of variation was >25% in samples from the variable group (see section 3.3.3.3 for definition of groups) (Table 3.21).

Table 3.21: Estimates of test reproducibility for the IDEIA and RIDASCREEN norovirus ELISA based on samples with positive, negative and discordant/variable ELISA/PCR results.

	Positives			Negatives			Variable results		
	Mean O.D	S.D	CV %	Mean O.D	S.D	CV %	Mean O.D	S.D	CV %
IDEIA	1.3	0.24	18%	0.082	0.0047	5.7%	0.196	0.1	51%
RIDASCREEN	1.7	0.41	24%	0.058	0.011	18%	0.16	0.052	33%

CV= coefficient of variation, S.D = standard deviation, O.D = optical density.

3.4.3.7 RIDASCREEN and RIDAQUICK results in a clinical context.

Among hospitalised children the severity of disease was higher in children with both positive PCR and immunological tests compared with children with a positive PCR test alone. The mean severity scores were, for the immunological test positive/negative children, 10.6/8.1 (p=0.03) for the RIDAQUICK and 10.6/8.8 (p=0.08) for the RIDASCREEN. Among hospitalised children the detection of norovirus by immunological tests was not affected by the timing of sample collection. The mean duration of diarrhoea at time of sample collection in those with norovirus detected by PCR were, for the immunological test positive/negative children, 4.2/3.4 days (p=0.18) for the RIDAQUICK and 3.8/4.6 days (p=0.27) for the RIDASCREEN (Table 3.22/3.23). Both the RIDASCREEN and RIDAQUICK tests detected norovirus for up to 13 days following the onset of diarrhoea (Figure 3.8).

Table 3.22: Relationships between RIDASCREEN ELISA result, disease severity (modified Vesikari score) and duration of diarrhoea within hospital samples positive for norovirus by PCR.

	RIDASCREEN Positive (n=46)	RIDASCREEN Negative (n=20)	P value
Disease severity (S.D)	10.6 (3.9)	8.8 (3.6)	0.08
Time since diarrhoea onset (S.D)	3.8 days (2.7)	4.6 days (2.7)	0.27

Table 3.23: Relationships between RIDAQUICK ICG result, disease severity (modified Vesikari score) and duration of diarrhoea within hospital samples positive for norovirus by PCR.

	RIDAQUICK Positive (n=52)	RIDAQUICK Negative (n=14)	P value
Disease severity (S.D)	10.6 (3.8)	8.1 (3.5)	0.03
Time since diarrhoea onset (S.D)	4.2 days (2.8)	3.4 days (2.1)	0.39

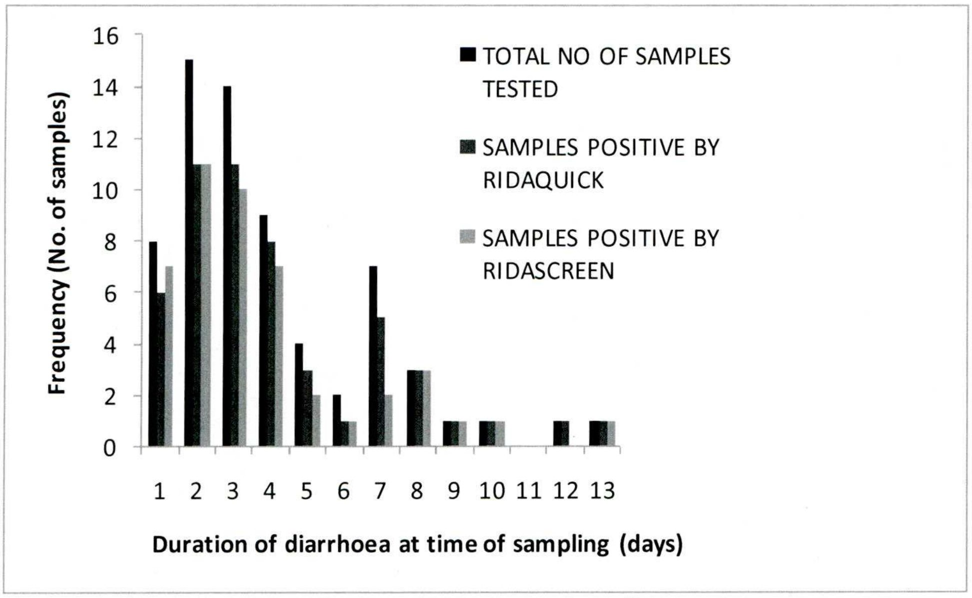


Figure 3.8: Sensitivity of the RIDASCREEN and RIDAQUICK tests with respect to the duration of diarrhoea at the time samples were collected within the hospital cohort.

3.4.3.8 Summary of results

IDEIA ELISA

Sensitivity = 49%

Specificity was 100%, in the entire cohort as well as all subgroups analysed.

Sensitivity subgroups: 62% in the hospital cohort

20% in the community cohort

0% in the genogroup I subgroup

55% in the genogroup II subgroup

Sensitivity at 80% specificity (ROC analysis) 67% in the entire cohort

58% in the community cohort

75% in the hospital cohort

Coefficient of variation positive samples: 18%

negative samples: 5.7%

variable samples: 52%

RIDASCREEN ELISA

Sensitivity = 64%, significantly higher than the IDEIA ELISA (p = 0.0013).

Specificity was 100%, in the entire cohort as well as all subgroups analysed.

Sensitivity in the subgroups: 70% in the hospital cohort

50% in the community cohort

12.5% in the GI subgroup

67% in the GII subgroup

The RIDASCREEN ELISA was significantly more sensitive than the IDEIA ELISA in the community (p = 0.0039) and genogroup II subgroups (p = 0.0073).

Sensitivity at 80% specificity (ROC analysis) 78% in the entire cohort

56% in the community cohort

91% in the hospital cohort

Coefficient of variation positive samples: 24%

negative samples: 18%

variable samples: 33%

RIDAQUICK ELISA

Sensitivity = 69%, significantly higher than the IDEIA ELISA ($p = 0.00002$)

Specificity was 100%, in the entire cohort as well as all subgroups analysed.

Sensitivity in the subgroups: 79% in the hospital cohort

47% in the community cohort

0% in the GI subgroup

76% in the GII subgroup

The RIDAQUICK ELISA was significantly more sensitive (significance at $p =< 0.05$) than the IDEIA ELISA in the hospital, community and genogroup II subgroups.

3.5 Discussion

Tests for norovirus infection have utility in two principal areas, for the completion of molecular epidemiological studies and the diagnosis of infection. The requirements of tests in these areas are different, therefore the discussion below assess the tests usefulness in these two areas separately.

3.5.1 Molecular epidemiological studies.

Studies of the molecular epidemiology of norovirus require processing of DNA products from PCR reactions. Obtaining these products by completing PCR on all samples under evaluation is time consuming and expensive. Given the low prevalence of norovirus in most studies, about 15% (Koopmans 2008), the costs and time involved may be reduced if samples are first tested using a simpler, cheaper method. ELISA may offer a relatively low cost/low technology test to analyse samples for norovirus infection. For an ELISA test to be suitable for pre-PCR testing it must be sufficiently sensitive. Of the two commercially available ELISAs the RIDASCREEN ELISA, in this study, was significantly more sensitive than the IDEIA ELISA. The RIDASCREEN ELISA should be the ELISA of choice if this method is to be used to test samples for norovirus. The sensitivity of the RIDASCEEN ELISA, at 70% in the hospital cohort, is still a lot less sensitive than PCR detection of norovirus. It was found that if the specificity of the RIDASCREEN test was adjusted to 80%, then the sensitivity in the hospital cohort increased to 90%. This may be an acceptable sensitivity and specificity for the test to be used prior to PCR in molecular epidemiological studies of norovirus.

3.5.2 Diagnostic use of norovirus tests

A diagnosis of norovirus infection allows completion of clinical epidemiological studies and a diagnosis to be given to an individual patient. Making a diagnosis of norovirus infection is difficult as there are many causes of gastroenteritis. The similarity of norovirus gastroenteritis, outside an outbreak, to other causes of gastroenteritis e.g. rotavirus, is likely to prevent a high pretest probability of norovirus infection from favourably effecting the post test probability of norovirus infection (a high pre test probability can raise the positive predictive value of a test). Also, the presence of norovirus in faeces is not clearly correlated with current illness as norovirus may be present in faeces for a prolonged period following infection. That is, a test for norovirus may have a good analytical accuracy but poor clinical/diagnostic accuracy. Analytical accuracy in this contest is the ability to detect norovirus in a clinical sample when norovirus is truly present in the sample. It does not give information on whether norovirus is causing disease. Clinical/diagnostic accuracy is the ability to detect norovirus which is causing disease. Measurements of analytical/clinical accuracy can be limited by an imperfect reference standard, the reference standard providing the information as to whether an organism/disease is truly present. In view of the knowledge that PCR provides an imperfect reference standard for the detection of norovirus disease, as it detects asymptomatic shedding of norovirus, an attempt improve the reference standard was made. An improved reference standard would allow better estimations of the tests predictive values and therefore a better understanding of their usefulness. An attempt to improve the reference standard, norovirus PCR, was carried out by estimating the detection of asymptomatic norovirus shedding. Estimates were that PCR detected asymptomatic shedding at 8% by PCR and 2% by ELISA (section 3.3.3.2). Based on these figures the PPV of PCR and ELISA for norovirus testing in this study was estimated. PCR on these assumptions had a PPV of 52% compared to a PPV of 83% by the RIDASCREEN ELISA. If the assumptions made in reaching this value are valid, PCR has limited value for norovirus infection in this study setting and RIDASCREEN ELISA based testing may offer a better diagnostic test than PCR. These estimates of PPVs are though, just estimates. There are clearly limitations of these estimates and this highlights the importance of including control groups in designing evaluations of diagnostic tests. In addition, the false negative results by the RIDASCREEN/RIDAQUICK tests were associated with a less severe disease. This supports ELISA/ICG based norovirus testing because missing a diagnosis of norovirus where the gastroenteritis is less severe may have more limited clinical implications.

The reproducibility of the ELISAs was good when the tests were positive/negative by PCR and ELISA on initial testing. Where discordant results were seen a repeated result had up to a 50% variation in the O.D value. These discordant results gave O.D readings close to the tests cut off values. Therefore, if a result is important and the O.D value is within 50% of the test cut off, the test result should be confirmed by a molecular test.

The specificity of the tests, 100%, is consistent with the manufacturer's findings. This appears a high sensitivity but consideration should be given to the reporting algorithm of this study. This study did not report a negative sample by PCR positive by ELISA unless repeat PCR testing was negative. Previous evaluations of ELISAs which report specificities below 100% have found most apparent false positives by ELISA are positive by PCR when retesting by an alternative PCR is carried out (Gray et al. 2007).

The clinical utility of making a diagnosis of norovirus is reduced by the lack of a specific therapy for norovirus. However, therapy and investigations for gastroenteritis may be reduced if a specific diagnosis is made. There may also be utility when investigating an outbreak of infection, as identifying the aetiological cause of an outbreak will direct the management of that outbreak. These benefits may be increased if the diagnosis can be made more rapidly.

This study shows the RIDAQUICK test to be sufficiently sensitive and specific as to be clinically useful in making rapid diagnoses of norovirus infection. This may be particularly valuable in the investigation of an outbreak as early responses to norovirus outbreaks have been associated with a reduction in the size of the outbreak (Lopman et al. 2004). Both RIDASCREEN and RIDAQUICK tests have the advantage that they can be used in laboratories without the facility for PCR, thus expanding the locations norovirus can be diagnosed.

Whilst immunological tests should be used within the first 72 hours of infection, this study demonstrated the RIDASCREEN and RIDAQUICK tests were able to detect norovirus for up to 13 days following infection. Although the numbers tested beyond seven days was low, these results demonstrate in patients with prolonged diarrhoea, these tests may still be used as a first line test to investigate for norovirus infection.

3.6 Limitations

- PCR diagnosis was used as an imperfect reference standard in this study.
- No control group was available to accurately define the prevalence of norovirus infection in the study group which would allow accurate calculation of the tests predictive values.
- The normalised values used to define the ROC curves were based on a single negative control per ELISA run.
- The data collected from the community cohort differed from the hospital cohort preventing direct comparisons. The duration of diarrhoea at the time of sample collection was not calculable from the data for the community cohort.

- The severity score used was developed for rotavirus and not specific for norovirus.
- A small number of Genogroup I samples were detected in the study making it difficult to comment on the tests usefulness to detect Genogroup I infections.

3.7 Further work

Improving the ELISA assay

To improve the assay for use as a screening tool prior to molecular testing it would be good to increase the sensitivity of the assay. The assays sensitivity could be increased if it could differentiate false negatives and true negatives with high negative O.D. values. There are a number of factors which affect the sensitivity and specificity of ELISAs including: concentration of capture antibody, duration of incubation, concentration of sample assayed and temperature of incubation. Investigating variations in these conditions may improve assay characteristics.

Improving the ICG assay

The RIDAQUICK ICG assay is marketed as a rapid diagnostic test, suggesting a 15 minute test time. This excludes the time it takes for reagents to achieve room temperature. In this study the reagents took at least 1 hour to reach room temperature. Where the arrival of samples cannot into a laboratory cannot be predicted this may result in a less rapid test or a test used outside the recommended experimental protocol. It will therefore be of benefit to know what impact completing the test with reagents below room temperature has and if there are methods which can speed the warming of reagents to room temperature without adversely affecting test performance.

Differentiating symptomatic infection from asymptomatic shedding.

To better understand the diagnostic value of the norovirus tests a reliable gold standard test is required. Recently, a PCR cycle threshold has been suggested for genogroup II norovirus by RT-PCR. This requires an individual laboratory to complete a ROC analysis of cases and controls, not possible for most laboratories (Phillips et al. 2009). In addition the viral load excreted between viruses is different with GII.4 excreting 100x more virus than other GII viruses and GII viruses excreting 100x more virus than GI viruses (Chan et al. 2006). An alternative method is to use the immunological response to differentiate acute infection from non acute infection. A rise in salivary IgA has been shown to develop in norovirus infection (Lindesmith et al. 2003). This would require acute and convalescent samples to be taken, which is not practical for many diagnostic studies. An alternative would be to measure IgA using an absence to indicate acute infection. This could be difficult as people may have antibody detectable from past infections or recent unsuccessful viral challenge. Diagnosing norovirus infection will therefore continue to be difficult until there is a greater understanding of norovirus shedding, sufficient to be able to differentiate it from symptomatic infection. In the first instance, ELISAs should be used on a series of symptomatic and asymptomatic patients to see if the asymptomatic infected patients are detected by commercial ELISA testing. This may not be the case given asymptomatic infection is associated with reduced levels of antigen excretion (Okhuysen et al. 1995).

Multicentre evaluation. The ELISA/ICG norovirus tests require evaluation in multiple centres and in different patient cohorts to establish test characteristics under different situations.

3.8 Conclusions

The RIDASCREEN norovirus ELISA offers a good preliminary screening tool for molecular epidemiological studies into norovirus in hospitalised children. They are also suitable to inform surveillance systems of the prevalence of norovirus in low and middle income countries. Both the RIDASCREEN and the RIDAQUICK norovirus tests offer a sensitive and specific diagnostic test for norovirus infection in hospitalised children. These tests may have a better positive predictive value than PCR when used as a diagnostic test.

CHAPTER 4

Molecular epidemiology of norovirus in Merseyside, UK and Aracaju, Brazil.

4.1 Introduction: Molecular epidemiology of norovirus

Norovirus is separated into five genogroups, of these genogroups I, II and occasionally genogroup IV cause infection in humans (Green et al. 2000). Within these genogroups are over 30 genotypes. Initial analysis of the molecular epidemiology of norovirus outbreaks showed genetic diversity in norovirus isolated from epidemiologically unrelated outbreaks (Ando et al. 1995). More recently the genogroup II, genotype 4 (GII.4) norovirus has been recognised as the cause of most cases of norovirus globally. Whilst there is heterogeneity within GII.4 in unrelated outbreaks of norovirus it has also been shown that highly similar strains of norovirus can be detected in epidemiologically unrelated outbreaks. A study by Noel *et al* on samples collected from 1993-1997 showed that multiple epidemiologically unrelated outbreaks from across the United States of America showed 100% nucleotide (nt) identity (Figure 4.1(Noel et al. 1999)) when a 277 bp sequence from the 5 prime end of ORF2 from norovirus was analysed. Further analysis in this study of the entire capsid sequence (1620 nt) of ten isolates showed half had below 10 base pair mutations different between strains. Given the mutation rate in norovirus has been estimated at 0.0043 nucleotide substitutions/site/year by BoK et al, over a year, 7 mutations might be expected to occur in this 1620bp region. This suggests many outbreak strains identified were related to each other by a recent ancestor. Since then, studies have repeatedly shown genetically similar GII.4 norovirus causing outbreaks intra/inter nationally (Vainio and Myrmel 2006; Tu et al. 2008; Ramirez et al. 2009; Sdiri-Loulizi et al. 2009; Siebenga et al. 2009). It has also been suggested GII.4 norovirus undergoes genetic drift over time (Gallimore et al. 2007; Siebenga et al. 2007). Antigenic drift results in new norovirus which are antigenically distinct from previous norovirus strains making the human population susceptible to infection by these new strains. This high degree of genetic variability seen within norovirus allows information on the epidemiology of norovirus to be obtained by analysis of its genetic sequence. Therefore, to understand this molecular epidemiology more, we have carried out an analysis of the genetic sequences from the studies into norovirus in hospitalised adult patients in the UK (section 4.2) and in community onset gastroenteritis in Brazilian children (section 4.3).

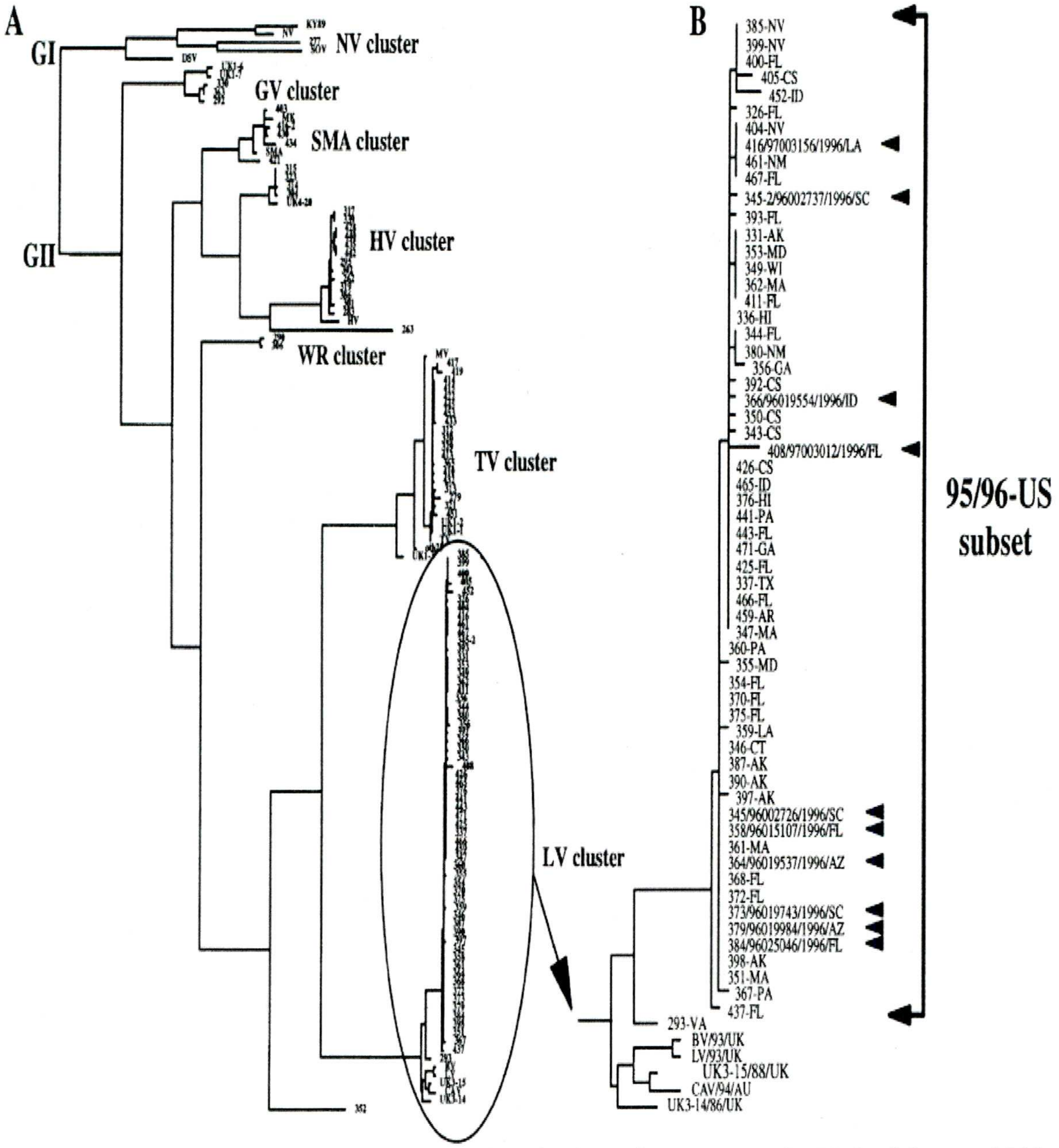


Figure 4.1: Phylogenetic analysis of norovirus isolates from across the United States, 1993-1997. Analysis based on the 277-base nucleotide sequences from the partial capsid region. Table reproduced from Noel *et al* (Noel et al. 1999).

4.2.1 Background: Molecular epidemiology of norovirus infection in hospitals

Most UK outbreaks of norovirus occur in health care settings, approximately 80%, and these health care related outbreaks occur mainly in the winter (Lopman et al. 2003). Research into the molecular epidemiology of health care associated norovirus has demonstrated that a single genotype (GII.4) is responsible for most outbreaks of infection. A number of studies have examined the molecular epidemiology of GII.4, principally within hospitalised patients. A study by Maguire *et al* sequenced a 270bp polymerase sequence of norovirus from outbreaks in East Anglia, UK (1996-7). This showed that mostly, distinct viral strains were identified in specific geographic locations with nearby geographical locations having genetically similar but distinct strains. The mutations which were associated with a particular location were often point mutations and infrequently associated with alterations in the amino acid functional group (Maguire et al. 1999). Similar results were found by Schreier *et al* (Schreier et al. 2000). Dingle *et al* investigated norovirus molecular epidemiology in nosocomial outbreaks of norovirus in Oxford, UK (2002-3) by sequencing a 3225 nucleotide section of norovirus. They found that a norovirus outbreak with clinical evidence of person to person transmission had accumulation of multiple mutations where outbreaks with evidence of a point source of infection were associated with single mutations (Dingle 2004). Evidence of intra-outbreak sequence homology, without reference to the suspected type of transmission, was shown by Xerry *et al* (Xerry et al. 2008; Xerry et al. 2009).

4.2.2 Introduction: Molecular epidemiology of norovirus in Merseyside, UK

The control of norovirus infection within health care institutions requires an understanding of its molecular epidemiology. To study this, norovirus isolates from the study into the oral diagnosis of norovirus, Chapter Two, were sequenced. The study was based in five hospitals in Merseyside, UK. The hospitals locations are shown in Figure 4.2. Patients in hospital with gastroenteritis were entered into the study. Full details of the participants are presented in section 2.3.1. An investigation of the molecular epidemiology of norovirus was a secondary research objective of the study into the oral diagnosis of norovirus, detailed in Chapter Two. Therefore there are limitations on the epidemiological data collected including the complete history of patient's locations within hospitals and probable transmission routes between patients not being collected.

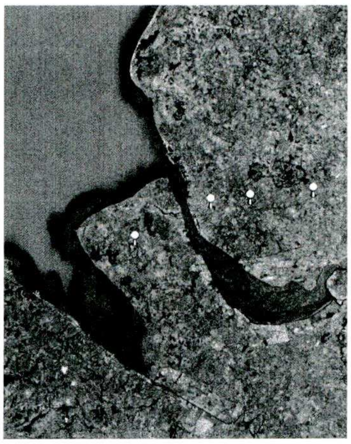


Figure 4.2: Location of the study sites for the oral diagnosis of norovirus study. Left to right, with distances from Arrow Park, the study sites are: Arrow Park, Royal Liverpool (9.8km), Broadgreen and Liverpool Heart and Chest (14.2km) and Whiston Hospital (21.3km).

4.2.3 Aims

To define the molecular epidemiology of norovirus infection in Merseyside hospitals, UK, during the winter of 2008/9: in particular:

- To define if there is evidence for inter-hospital transmission of norovirus in Merseyside hospitals, UK, during the winter of 2008/9.
- To define if introduction of norovirus into Merseyside hospitals is a single or recurrent event.
- To define if there is evidence specific geographical areas within Merseyside have distinct strains of norovirus.

4.2.4 TEST METHODS

Qualitative reverse transcriptase PCR: See 2.3.3.1.

DNA purification: See 2.3.3.2.

Sequencing of PCR products: See 2.3.3.3.

Processing and aligning sequence data: See 2.3.3.4.

4.2.5 Results

A total of 100 people were consented for this study with all providing oral samples and 66 providing faecal samples. A total of 22 oral samples and 59 faecal samples had norovirus sequence detected by real time RT-PCR. All oral samples and a representative selection of these faecal samples underwent qualitative PCR to obtain PCR product for sequencing. A total of 7 oral and 44 faecal samples were successfully sequenced. Within these norovirus sequences were 8 distinct strains, A-H, with a number of strains identified in multiple patients, Figure 4.3 and Table 4.1. The strains, based on a 278bp region of norovirus sequence, mainly varied by only 1-3 base pairs, Figure 4.4.

There were 3/100 patients in this study who were admitted to hospital with gastroenteritis, all other patients acquired the disease nosocomially. Of these 3 patients, only 1 (RH8B-12/08(16) (see Table 4.1) provided a faecal sample for testing. This patient`s (RH8B-12/08(16)) strain (Strain H) of norovirus was distinct from all other sequences identified in the study.

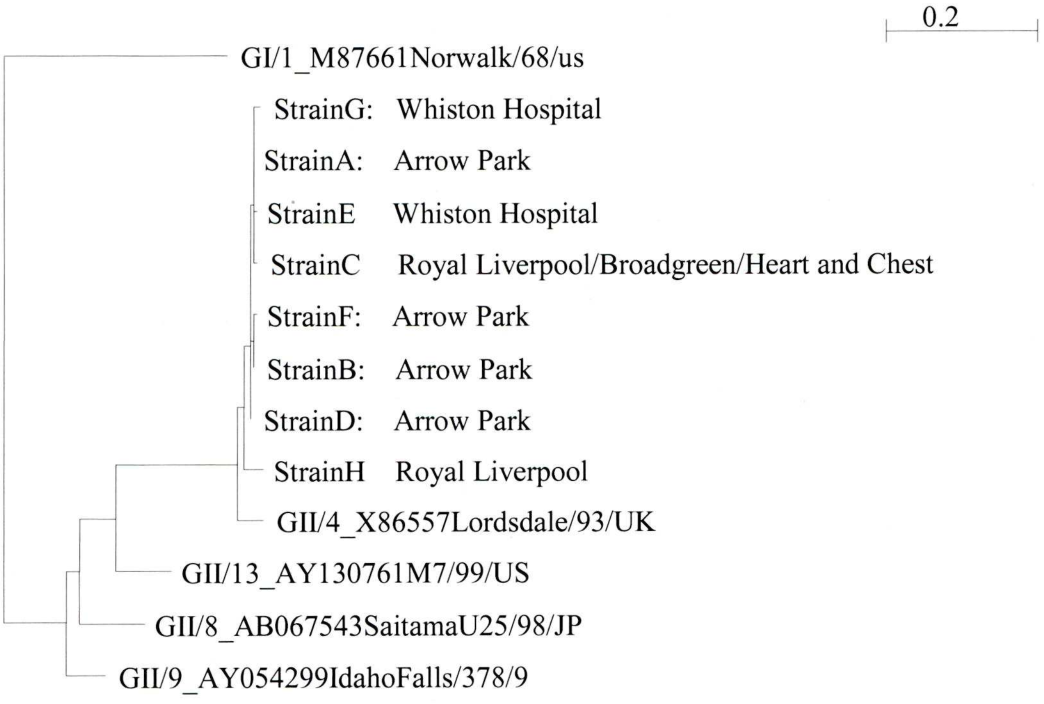


Figure 4.3: Phylogenetic alignment of the strains identified in the oral diagnosis of norovirus study, A-H, and the hospitals each strain was identified in. Analysis based on 278bp region of ORF2 (starting from ORF2 start codon). Phylogenetic analysis conducted using neighbour joining method. The calibration represents nucleotide substitutions per base.

Table 4.1: The patients in the study into the oral diagnosis of norovirus listed alongside the strains of norovirus identified in this study, A-H.

Strain	**Patient identifying label**
Strain A (n=14)	AP33-17/12/08(31), AP43-09/01/09(52), AP43-08/01/09(53), AP24-11/01/09(56), AP24-11/01/09(57), AP24(MW)-12/01/09(60), AP24-10/01/09(63), AP43-15/01/09(74), AP27-14/01/09(79), AP26-20/01/09(103), AP27-19/01/09(105), AP27(MW)-19/01/09(106), AP27-21/01/09(109), AP38-21/01/09(110)
Strain B (n=9)	AP33-14/12/08(30), AP43-08/01/09(50), AP23-13/01/09(65), AP23(MW)-13/01/09(65), AP43-13/01/09(75), APAMAU-14/01/09(76), AP37-17/01/09(85), AP38-17/01/09(87), AP38-14/01/09(88), AP38-21/01/09(110)
Strain C (n=12)	BG5-25/12/08(38), BG5(MW)-25/12/08(38), RH9X-27/12/08(41), RH9X-28/12/08(42), RH3X-24/12/08(46), BG5-28/12/08(47), BG5-08/01/09(54), BG5-10/01/09(55), BG5(MW)-10/01/09(55), BG5-12/01/09(71), BG11-17/01/09(94), BG11-17/01/09(96), CTA-30/01/09(121), CTA-31/01/09(122)
Strain D (n=4)	AP37-23/11/08(3), AP37-22/11/08(4), AP37-22/11/08(10), AP37-18/11/08(11)
Strain E (n=3)	WHF1-08/12/08(22), WHF1-26/11/08(23), WHB2-0912/08(28)
Strain F	AP33-17/12/08(33)
Strain G	WHC3-22/11/08(6)
Strain H	RH8B-10/11/08(16)
No strain designation	AP26(MW)-20/01/09(102), BG11(MW)-17/01/09(94), AP37-25/11/08(9)

Patient's identification: location, date of onset of symptoms and study number i.e. Hospital and Ward-DD/MM/YY(study number)). AP= Arrow Park; BG = Broadgreen Hospital; RH=Royal Liverpool Hospital; CT=Liverpool Heart and Chest Hospital and WH= Whiston Hospital. Mouthwashes are identified by MW (mouthwash) Where sequence information was incomplete no strain designation was assigned.

```
                5         15         25         35         45         55
StrainA    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainB    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainC    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainD    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainE    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainF    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA [illegible]CTCGTCCCA
StrainG    ATGAAGATGG CGTCGAATGA CGCCAACCCA TCTGATGGGT CCGCAGCCAA CCTCGTCCCA
StrainH    ATGAAGATGG CGTCGA[illegible]TGA CGCCAACCCA TCTGATGGGT CC[illegible]CAGCCAA CCTCGTCCCA

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
               65         75         85         95        105        115
StrainA    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainB    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainC    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainD    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGT[illegible]G GTGCCGCTAT TGCGGCGCCT
StrainE    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainF    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainG    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGTCG GTGCCGCTAT TGCGGCGCCT
StrainH    GAGGTCAACA ATGAGGTTAT GGCTTTGGAG CCCGTTGT[illegible]G GTGCCGC[illegible]AT TGCGGC[illegible]CCT

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              125        135        145        155        165        175
StrainA    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA ATAATTTTGT ACAAGCCCCT
StrainB    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA A[illegible]AATTTTGT ACAAGCCCCT
StrainC    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA ATAATTTTGT ACAAGCCCCT
StrainD    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA ATAATTTTGT ACAAGCCCCT
StrainE    GTAGCGGGCC AACAAAATGT AAT[illegible]GACCCC TGGATTAGAA ATAATTTTGT ACAAGCCCCT
StrainF    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA A[illegible]AATTTTGT ACAAGCCCCT
StrainG    GTAGCGGGCC AACAAAATGT [illegible]ATTGACCCC TGGATTAGAA ATAATTTTGT ACAAGCCCCT
StrainH    GTAGCGGGCC AACAAAATGT AATTGACCCC TGGATTAGAA A[illegible]AATTTTGT ACAAGCCCCT

           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              185        195        205        215        225        235
StrainA    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC
StrainB    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC
StrainC    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACT[illegible]TG GAGCGCGCCC
StrainD    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC
StrainE    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC
StrainF    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC
StrainG    GGTGGAGAGT TCACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGC[illegible]CCC
StrainH    GGTGGAGAGT T[illegible]ACAGTATC CCCTAGAAAC GCTCCAGGTG AAATACTATG GAGCGCGCCC

           ....|....| ....|....| ....|....| ....|....| ....|..
              245        255        265        275        285
StrainA    TTAGGCCCTG ATCTGAATCC CTACCTATCT CATTTGGCCA GAATGTA
StrainB    TTAGGCCCTG ATCTGAATCC CTACYTATCT CATTTGGCCA GAATGTA
StrainC    TTAGGCCCTG ATCTGAA[illegible]CC CTACCTATCT CATTTGGCCA GAATGTA
StrainD    TTAGGCCCTG ATCTGAATCC CTACCTATCT CATTTGGCCA GAATGTA
StrainE    TTAGGCCCTG ATCTGAATCC CTACCTATCT CATTTGGCCA GAATGTA
StrainF    TTAGGCCCTG ATCTGAATCC CTACCTATCT CATTTGGCCA GAATGTA
StrainG    TTAGG[illegible]CCTG ATCTGAATCC CTACCTATCT CATTTGGCCA GAATGTA
StrainH    TTAGGCCCTG AT[illegible]TGAATCC CTACCT[illegible]TC[illegible] CATTTGGCCA GAATGTA
```

Figure 4.4: Sequence data from ORF2 of norovirus for strains, (A-H), identified in the oral diagnosis of norovirus study. The reference strain is Strain A and where sequence differs from Strain A the base is highlighted.

4.2.6 Discussion

Norovirus infection in Merseyside hospitals in the winter of 2008/9 was caused by GII.4 norovirus, GII.4 being recognised as the principal cause of nosocomial norovirus globally. The 8 distinct strains detected in this study were associated with specific geographical areas. Strains A, B, D and F were seen only in Arrow Park Hospital, Upton, Wirral. Strains E and G were seen in only Whiston Hospital, Prescot. Strain C was seen in more than one hospital: Royal Liverpool, Broadgreen and Liverpool Heart and Lung hospital. The hospitals with strain C serve a population living in the same location, transfer patients frequently between themselves and are geographically close to each other, approximately 4kms apart. It is therefore possible Strain C was present in the community which these hospitals serve or spread between these hospitals. These findings are consistent with those of Maguire *et al* (Maguire et al. 1999) who also found a predominance of distinct viral strains in specific but closely located geographical areas. The detection of distinct strains within specific locations may represent either each location has its own distinct strains of norovirus, or, that there is such a large degree of variation within norovirus in the community that it is unlikely the same strain of norovirus would be detected in two different locations.

The detection of a single strain on multiple wards over a period of a month is difficult to interpret. It initially suggests environmental contamination but a similar finding, distinct strains in multiple outbreaks, was noted by Dingle *et al*, after analysing a 285nt RNA polymerase region in multiple outbreaks (Dingle 2004). Further analysis, of a 3225nt section of norovirus sequence, allowed the investigators to subsequently define that distinct strains were responsible for single outbreaks. By resolving that the outbreaks were caused by different strains Dingle *et al* concluded the outbreaks resulted from multiple viral introductions from the community. The data at Arrow Park hospital, though not as detailed, is consistent with this conclusion as three different strains were found to cause outbreaks, Strains A, B and D. Further genetic analysis of our isolates is needed before making more detailed conclusions on the associations of outbreaks defined in this study as having the same strain. In addition, information on strains circulating in the community would help to confirm if the local community was the source of the strains of norovirus identified in the study.

Strain H had the most mutations present compared to the other strains. This strain was found in the eldest person in the study, 97 years old, who had also had the longest duration of symptoms at sampling, 23 days. This patient was admitted to the hospital with gastroenteritis therefore it is possible the virus was acquired with the genetic sequence we obtained. It is also possible that *in vivo* mutation occurred, as previously described (Nilsson et al. 2003). The environmental sites where mutations occur are important to understand as these mutations represent viral evolution. In theory, controlling the sites of this evolution would prevent the development of new viral strains for which humans have no immunity.

4.2.7 Limitations

- Epidemiological and sequence data was incomplete preventing more detailed analysis of closely related, in time and place, outbreaks of norovirus.
- The molecular analysis of the norovirus strains within these hospitals is limited by an absence of information on the strains circulating in the community in Merseyside. For example, why was only the GII.4 strain of norovirus detected in this study? Is the GII.4 strain of norovirus biologically adapted to transmission in hospitals or is it predominant in the community?

4.2.8 Further work

- Further sequence analysis of distinct strains is needed to increase confidence in the study findings
- Analysis of the clinical and molecular epidemiology of community acquired norovirus infection is needed to define the source/s of community acquired norovirus infection.

4.3.1 Background: Molecular epidemiology of community acquired paediatric norovirus infection

The molecular epidemiology of paediatric norovirus infection has been investigated globally and both genogroup I and genogroup II norovirus commonly cause infection. One genotype, GII.4, is the predominant cause of norovirus infection. Other genotypes that are more common include GII.2/3/5/7 (Dove et al. 2005; Kirkwood et al. 2005; Sdiri-Loulizi et al. 2009). Whilst GII.4 is the predominant genotype it does not usually dominate the epidemiology of paediatric diarrhoea in the way that it does in nosocomial infection. Some studies show that GI and GII infections are detected equally as often, with the detection of multiple genotypes being common (Colomba et al. 2007; Monica et al. 2007; Soares et al. 2007; Victoria et al. 2007; Lee et al. 2008; Malasao et al. 2008; Rachakonda et al. 2008; Chhabra et al. 2009; Guo et al. 2009; Xavier et al. 2009).

4.3.2 Introduction: Molecular epidemiology of norovirus in Aracaju, Brazil.

Children are the age cohort mainly infected by norovirus. Given no vaccine is available to prevent norovirus infection, preventing transmission of norovirus is the main strategy available to prevent children becoming infected. An understanding of the molecular epidemiology of norovirus in this cohort may allow an improved understanding of transmission of norovirus. This may lead to prevention strategies being better informed and further research into norovirus transmission being appropriately designed. Therefore, a study into the molecular epidemiology of the norovirus isolates derived from the study into norovirus diagnostics, Chapter Three, on Brazilian paediatric faecal samples, was undertaken.

The study used faecal samples collected from children with acute gastroenteritis, Aracaju, Brazil. The population was made of two cohorts of children with gastroenteritis: Children presenting to hospital with gastroenteritis and children in the community with gastroenteritis who were part of a longitudinal study of investigating paediatric gastroenteritis. Full details of the participants are presented in section 3.3.1.

4.3.3 Aims

- To define the molecular epidemiology of community acquired norovirus infection in children in Aracaju, Brazil, in 2006/7 including:
- Defining the tempora-spatial distribution of distinct norovirus strains in Aracaju, Brazil, over the study period, October 2006-December 2007.

4.3.4 Methods: See section 4.2.4

4.3.5 Results

A total of 96 faecal samples had norovirus infection confirmed by real time and qualitative PCR. Of these, 79 samples produced sufficient PCR product to obtain sequence data. The phylogenetic profile of these isolates is shown in Figure 4.5. Most isolates were detected in genogroup II and most f these were GII.4. The molecular epidemiology of norovirus infection detected in the community and hospital cohorts did not obviously differ.

In addition a sequence of uncertain strain classification (genotype) was detected in patients 89/93. These unclassified sequences are shown in more detail in Figure 4.6. A number of sequences were identified in multiple patients, Table 4.2.

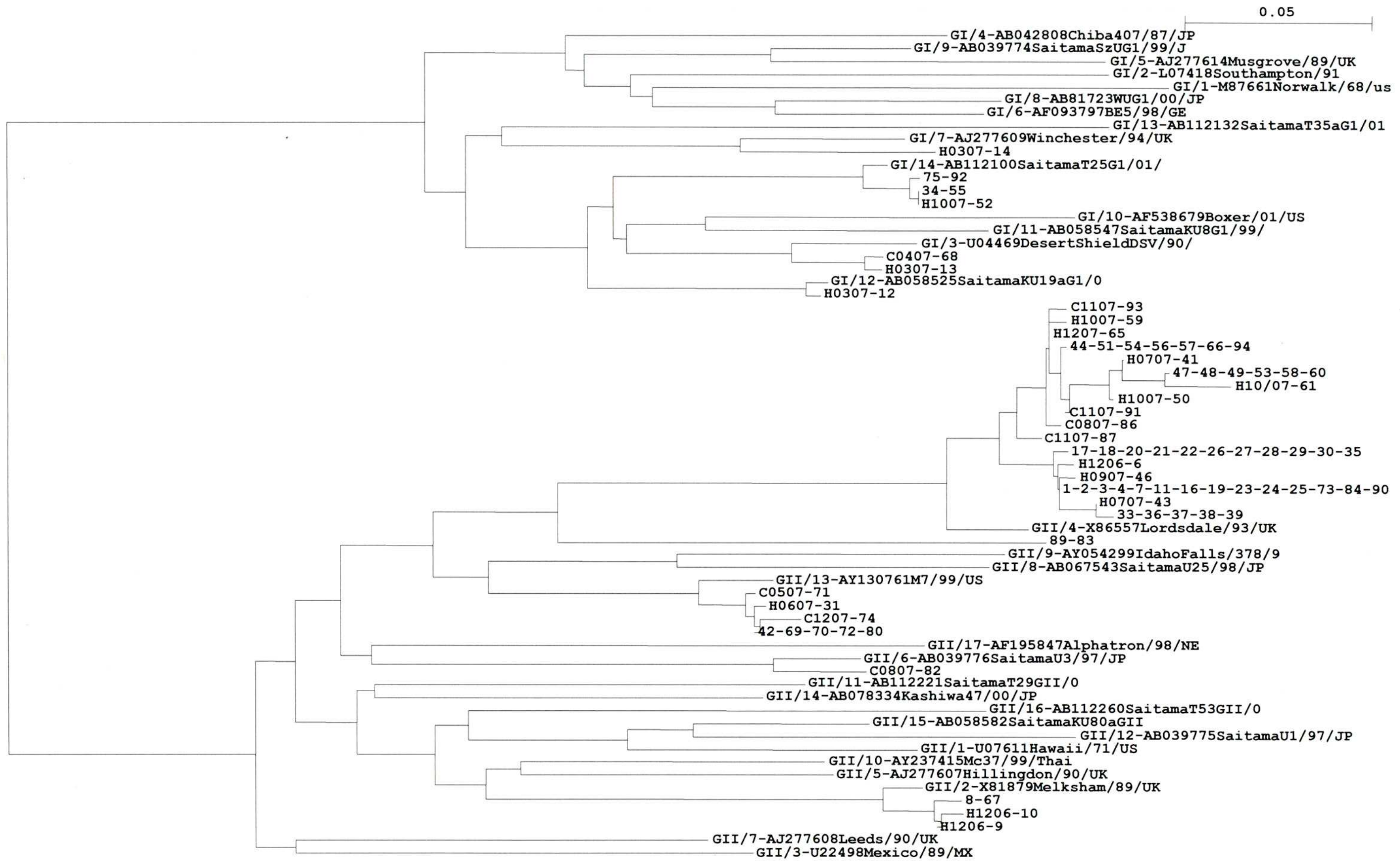


Figure 4.5: Phylogenetic analysis (NJ method) of norovirus isolates from children with gastroenteritis, Aracaju, Brazil. Analysis conducted on 278bp region of ORF2. Patients labels: H, hospital, C, community, MonthYear of isolation and study number e.g. H0607-43. Where multiple isolates with same position on the tree present only the study number is given e.g. 10-25-44 for study numbers 10, 25 and 44 (Table 4.2).

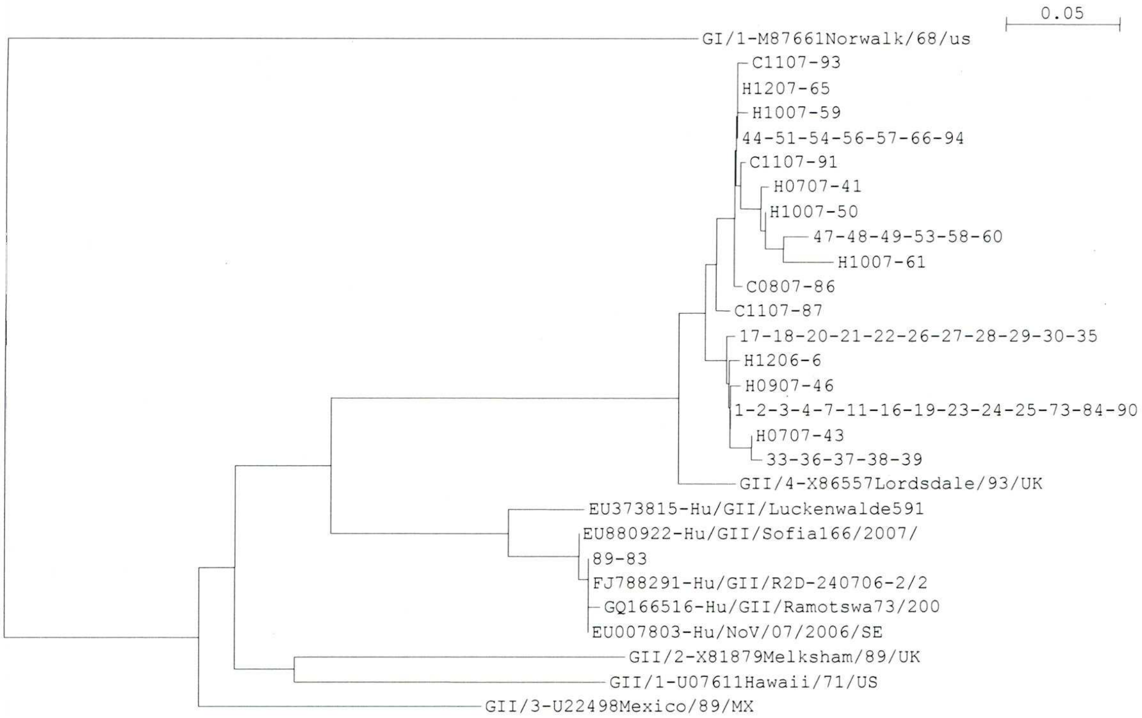


Figure 4.6: Phylogenetic analysis (NJ method) of GII norovirus and undetermined genogroup norovirus isolates from children with gastroenteritis, Aracaju, Brazil. Analysis carried out on an approximately 278bp region of ORF2. This study patients are labelled as H, hospital, C, community, MonthYear of isolation and study number e.g. H0607-43. Where multiple isolates with same position on the tree are present just the study number is given e.g. 10-25-44 (Table 4.2). Genbank accession No's: EU007803.1, GQ166516, FJ88291 and FJ788291.1 are the isolates most closely related to Study No. isolates 83 and 89.

Table 4.2: Dates, sources and study numbers for isolates grouping at the same terminal branch in Figure 4.4.

Strain designation	Patient details (Study No.)	Time between first and last detection (months)
1	H10/06(1), H11/06(3), H11/06(2), H11/06(4), H12/06(7), H12/06(11),) H03/07(16), H04/07(19), H05/07(23), H05/07(24), H05/07(25), H06/07(30), C06/07(73), C06/07(84), C09/07(90)	12
2	H04/07(17), H04/07(18),H04/07(20),H04/07(21),H04/07(22), H05/07(27), H05/07(26), H06/07(28),H06/07(29), H06/07(30),H06/07(35)	3
3	H07/07(44), H10/07(51), H10/07(54), H10/07(56), H10/07(57), C10/07(94), H12/07(66)	5
4	H09/07(47), H09/07(48), H09/07(49), H10/07(53), H10/07(58), H10/07(60)	2
5	H07/07(42), C08/07(69), C05/07(70), C05/07(72), C01/07(75), C08/07(80)	7
6	H06/07(33), H06/07(36), H06/07(37), H07/07(38) H07/07(39)	2
7	C09/07(83), C10/07(89)	2
8	H06/07(34), H10/07(55)	4
9	C11/07(92), C01/07(75)	2
10	H12/06(8), NK(67)	nk

Patients label: community (C) or hospital location (H); date sample collected month/year(study number).

4.3.6 Discussion

The noroviruses identified in this study included both genogroups I and II. They were mainly genogroup II (91%: 70/77) and the main genotype was GII.4 (75%: 58/77). Two patients had mixed genogroup I and II infections. These data are consistent with other published studies that have shown GII, and principally GII.4 being the main genogroups/genotypes of norovirus detected (Soares et al. 2007; Victoria et al. 2007; Lee et al. 2008; Malasao et al. 2008; Rachakonda et al. 2008; Chhabra et al. 2009; Guo et al. 2009; Nayak et al. 2009; Ramirez et al. 2009; Xavier et al. 2009). In addition, a putative new genotype of norovirus only previously described four times was detected in this study. This was Strain 7, detected in two samples, C09/07(83), C10/07(89). The phylogenetic analysis of strain 7 showed the sequence distance between it and its closets reference genotype, GII.4, was similar to distances seen recognised genotypes (Figure 4.5). A nucleotide BLAST analysis of the sequence registered three human (Genbank accession No's: EU007803.1, GQ166516, FJ88291) and one surface water derived (FJ788291.1|) matching sequences (≥98% maximal identity) (Figure 4.6). The human isolates were detected in Sweden 2002-6; Bulgaria 2007 and Botswana 2006. The surface water sample was from Singapore in 2006. The next most similar sequence was 93% similar (EU373815). This strain is widely dispersed, now detected in four continents and may be a newly emerging strain. Continued molecular surveillance will be needed to investigate this possibility. It may also represent a new genotype but more genetic sequence data is needed to make that conclusion.

A number of distinct sequences (strains) were detected in multiple patients (Table 4.2). Of note, several strains were identified over prolonged time periods. Strain 1, 3 and 5 were detected over a period of 12, 5 and 7 months respectfully. The patients with these strains were resident over a wide area within Sergipe State and Bahia State in Brazil (Personnel communication: R Gurgel, Sergipe University, Brazil). With a short duration of infection, if person to person transmission maintained the virus over this time period then multiple transmission events (viral replications) would have been expected to occur over these time periods. Mutations associated with viral replication has been shown in three studies. In two cases of prolonged excretion mutation rates of 0.03 nucleotide substitutions per site per year were detected (Nilsson et al. 2003; Dingle 2004) and in one person to person outbreak a rate of 0.04 was detected (Dingle 2004). These mutation rates should result in 8 mutations in a 277bp region over a one year period (0.03x277). Given one strain was detected over a one year period without any mutations in the 277bp region, it is tempting to conclude this provides evidence that person to person transmission was not how norovirus spread in this community, and therefore transmission was based on environmental sources of norovirus. This conclusion cannot though be made as the 277bp region studied in these patients (5 prime end of ORF2) has a lower rate of mutation than other regions of the norovirus genome, and the mutation rate of this section of genome has not been reported (Bok et al. 2009). More detailed sequence analysis and epidemiological information is needed to understand how the GII.4 strains spread in this population. The recognition of the global distribution of similar strains of norovirus over prolonged periods has also been noted previously (Noel et al. 1999; Siebenga et al. 2009). Since that time a definitive answer has not emerged to explain how norovirus spreads globally. Speculated transmission routes include person to person and environmental sources including food (Widdowson et al. 2005) and water contamination (Lodder and de Roda Husman 2005; Maunula et al. 2005). Future epidemiological research may better define how norovirus spreads globally and this may allow the implementation of effective control strategies.

4.3.7 Limitations

- Only norovirus isolates with sufficient PCR product to sequence after a single qualitative PCR reaction were sequenced. This provided representatives from all outbreak locations in the oral diagnosis study and the majority (79/96) of samples from the Brazilian study. Therefore all norovirus infected patients from the studies did not have their isolates sequenced. This may have introduced some bias into the phylogenetic analysis of these two norovirus infected cohorts.

- Data on exposure history (to gastroenteritis) was not collected limiting ability to epidemiologically link subjects with molecularly related norovirus.
- Sequence data collected was limited to the shell domain of ORF2.

4.3.8 Further work

- Descriptive epidemiological studies are needed to describe environmental sources of norovirus infection and person to person transmission in this community.
- Further sequence analysis of Strain 7 is needed to investigate if it is a new genotype of norovirus.

4.4 Conclusions

This study has provided a preliminary assessment of the molecular epidemiology of norovirus infection in two very different settings. One setting a paediatric cohort of community acquired infection in Brazil, and the other setting a predominantly elderly cohort with nosocomially acquired infection. It is difficult therefore to bring these data sets together to provide a joint conclusion about the molecular epidemiology of norovirus infection, which may in fact be very different within these two cohorts. It is though clear that in both settings GII.4 norovirus is the main strain causing norovirus infection. Globally, spread of GII.4 norovirus has been shown to occur by many routes, from foodborne sources e.g. shellfish and salads, to water sources, with large outbreaks caused by municipal water supplies and from person to person. In the future it may be of benefit to the prevention of norovirus infection to understand if one of these methods alone is responsible for maintaining GII.4 transmission within populations, or if transmission is maintained by a combination of different transmission routes.

Conclusions

Conclusions

Evaluating diagnostic tests is a difficult process (Scott et al. 2008). Ideally any evaluation would have at its centre a gold standard which was 100% accurate. This is rarely the case in practise, and evaluations must proceed in their absence. This study evaluated novel approaches to the diagnosis of norovirus.

In Chapter Two, results from an investigation into the sensitivity of the oral diagnosis of norovirus infection were reported. The sensitivity was found to be 24% compared to diagnosis using faecal samples. The detection of norovirus was associated with vomiting and inversely associated with the duration of diarrhoea and vomiting. This chapter concludes, given the low sensitivity of oral samples, faecal samples should continue to be used to diagnose norovirus infection. It may though be of benefit to investigate swab samples of faeces and vomit as samples to diagnose norovirus infection, and this is ongoing.

In Chapter Three, the results of an investigation into new immunological norovirus tests, the RIDASCREEN ELISA and RIDAQUICK immunochromatographic tests, were reported. This showed the RIDASCREEN ELISA and the RIDAQUICK ICG had a sensitivity of 70% and 79% respectively in hospitalised children in Aracaju, Brazil. Both tests had a specificity of 100%. This was based on a comparison to PCR diagnosis of faecal samples. Given these findings, how should a laboratory decide on which test is best for them to use to diagnose norovirus infection? This may be carried out by considering practical factors. Practical factors include the laboratory situation testing is to be undertaken in. For instance, some laboratories may have no molecular diagnostic tests available and the choice will therefore depend on how many samples the laboratory anticipates testing. Thus, the RIDAQUICK test would be appropriate if low numbers are to be examined, the RIDASCREEN if larger numbers were being tested. In those laboratories with molecular testing available the number of tests being carried out should dictate which test to use. If small numbers are being tested, given the high specificity of immunological testing, samples could be first tested immunologically followed by PCR testing if needed. If laboratories are routinely testing many samples it may be preferable to use PCR. Samples positive by PCR could be categorised as true positives by either defining a cut off PCR value or confirming by the RIDASCREEN/RIDAQUICK, given the lower probability of a false positive by antigen detection. Another means of deciding between tests is to consider their clinical utility. Given there is no specific treatment for norovirus gastroenteritis there are many situations where a diagnosis will have no benefit to the patient. Without consideration of a test's clinical utility these tests will drift into becoming routine diagnostic tests without an understanding on the impact they have on patient care. In particular, the role norovirus diagnostics have in outbreaks of norovirus gastroenteritis needs further consideration before deciding how to confirm a norovirus outbreak. To decide upon the role norovirus diagnostics have in outbreaks of gastroenteritis two questions need answering. The first question is if there is actually a need for laboratory testing of norovirus. Clinical assessment of an outbreak of norovirus, in the experience of the Infection Control team at the Royal Liverpool University Hospital (RH) (personnel communication: G. Smith, RH), is an accurate means of diagnosing norovirus infection. This is also supported by the use of simple inclusion and exclusion criteria for the oral diagnosis study. This resulted in 89% of participants entered for this study having norovirus infection. Secondly, consideration should be given to the impact of laboratory testing in controlling norovirus outbreaks. Given these outbreaks require prompt instigation of infection control practices there is a risk that these actions are delayed whilst waiting for a norovirus test result. Therefore whether norovirus diagnostics contribute positively to the control of norovirus outbreaks should be confirmed.

In Chapter Four, the phylogenetic analysis conducted highlighted important issues regarding the transmission of norovirus. The principal issue raised was how norovirus transmission occurs and the results raised the possibility that environmental sources may be the cause of norovirus infection over a wide area in Brazil. In a globalised world it is easy to see how this may occur, with for example, food exported globally. Water and food are likely environmental reservoirs of infection. Contamination of water and food offers challenges and opportunities. The challenge results from the failure of established processes for cleaning water/food. The opportunity arises when norovirus is considered alongside other viral agents which cause infection via drinking water/food (Leclerc et al. 2002). Other agents include enteroviruses, astrovirus, rotavirus and hepatitis A and E. All these agents are RNA viruses, all are non enveloped and all are between 20-70nm in diameter. Therefore, there is the potential that devising/implementing methods stopping the transmission of any one of these viruses may prevent transmission of all of these viruses.

In conclusion, the analytical sensitivity of norovirus immunological diagnostics has increased with the introduction of the RIDASCREEN and RIDAQUICK norovirus diagnostic tests. The clinical accuracy though, of both tests, remains uncertain. Establishing these relationships offers challenges for future researches of immunological norovirus diagnostics. Given the lack of preventative strategies for norovirus infection globally, further work into understanding the global transmission of epidemic strains of norovirus is needed.

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Appendix

Research ethics approval: Oral diagnosis of norovirus study

Bolton Research Ethics Committee
Room 181, Gateway House
Piccadilly South

M60 7LP

Telephone: 0161 237 2585
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13 March 2008
Manchester

Private & Confidential
Dr A Kirby, Clinical Lecturer in Microbiology
Department of Infection and Host Defence
Liverpool University
8th Floor, Duncan Building
Daulby Street
Liverpool
L69 3GA

Dear Dr Kirby

Full title of study: **Oral diagnosis of norovirus infection**
REC reference number: **08/H1009/12**

Thank you for your response to the Committee's request for further information on the above research and for submitting revised documentation. The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA) There is no requirement for other Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. **Please note in particular the requirements relating to the submission of progress and other reports in points 4 and 10.**

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
Application	5.5	25 January 2008	
Investigator CV	Nigel Cunliffe		
Investigator CV	Andrew Kirby		
Protocol	3.0	03 March 2008	
Compensation Arrangements	1	28 January 2008	
Participant Information Sheet: Consentee	3.0	03 March 2008	
Participant Information Sheet: Patient	3.0	03 March 2008	
Participant Information Sheet: Health care professionals	1.0	22 November 2007	
Participant Consent Form: Consentee	2.0	25 February 2008	
Participant Consent Form: Patient	2.0	25 February 2008	
Response to Request for Further Information	1	28 February 2008	
Response to Request for Further Information	2	03 March 2008	
Division of responsibilities	1	22 November 2007	
Opt-in/out slip	1.0	25 February 2008	

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review. Here you will find links to the following:

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Reports. Please refer to the attached Conditions of approval.
c) Amendments. Please refer to the attached Conditions of approval.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

08/H1009/12	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Frank Bowman
Chair

Table A1: Database: Oral diagnosis of norovirus study.

Study ID (Number)	Age	Sex	Ward	Date Of admission	Date of oral sampling/data collection	Duration of admission at oral sampling: Days	Date of Onset: Vomiting
AP37-11/08(3)	62	F	AP 37	18/11/2008	24/11/2008	6	23/11/2008
AP37-11/08(4)	47	M	AP 37	20/11/2008	24/11/2008	4	22/11/2008
AP37-11/08(2)	54	F	AP 37	20/11/2008	24/11/2008	4	19/11/2008
WHC3-11/08(7)	70	M	WH C3	20/11/2008	25/11/2008	5	22/11/2008
AP37-11/08(11)	74	F	AP 37	15/11/2008	25/11/2008	10	18/11/2008
WHC3-11/08(5)	54	F	WH C3	18/11/2008	25/11/2008	7	No vomiting
AP37-11/08(10)	66	F	AP 37	17/11/2008	25/11/2008	8	No vomiting
WHC3-11/08(8)	89	F	WH C3	16/11/2008	25/11/2008	9	No vomiting
WHC3-11/08(6)	84	M	WH C3	10/10/2008	25/11/2008	46	No vomiting
AP37-11/08(9)	42	M	AP 37	21/11/2008	25/11/2008	4	No vomiting
WHC3-11/08(13)	77	F	WH C3	02/10/2008	26/11/2008	55	23/11/2008
WHC3-11/08(14)	53	M	WH C3	23/11/2008	26/11/2008	3	23/11/2008
WHC3-11/08(12)	53	F	WH C3	21/11/2008	26/11/2008	5	25/11/2008
WHC3-11/08(15)	88	F	WH C3	22/11/2008	26/11/2008	4	26/11/2008
RH8B-12/08(16)	97	F	RH 8B	20/11/2008	03/12/2008	13	10/11/2008
WHF1-12/08(23)	83	F	WH F1	26/11/2008	11/12/2008	15	26/11/2008
WHB2-12/08(26)	81	F	WH B2	03/12/2008	11/12/2008	8	05/12/2008
WHF1-12/08(24)	85	F	WH F1	04/12/2008	11/12/2008	7	09/12/2008
WHB2-11/08(27)	78	F	WH B2	24/11/2008	11/12/2008	17	07/12/2008
WHF1-12/08(21)	83	F	WH F1	28/11/2008	11/12/2008	13	08/12/2008
WHF1-12/08(20)	73	F	WH F1	03/12/2008	11/12/2008	8	No vomiting
WHF1-12/08(22)	87	F	WH F1	18/10/2008	11/12/2008	54	No vomiting
WHB2-12/08(28)	78	F	WH B2	28/11/2008	11/12/2008	13	No vomiting
AP33-12/08(33)	71	F	AP 33	12/12/2008	18/12/2008	6	17/12/2008
AP33-12/08(32)	95	F	AP 33	10/12/2008	18/12/2008	8	17/12/2008
AP33-12/08(35)	72	F	AP 33	08/12/2008	18/12/2008	10	15/12/2008
AP33-12/08(34)	74	F	AP 33	10/10/2008	18/12/2008	69	15/12/2008
AP33-12/08(30)	67	F	AP 33	08/12/2008	18/12/2008	10	14/12/2008
AP33-12/08(31)	66	F	AP 33	16/11/2008	18/12/2008	32	No vomiting
BG5-12/08(39)	76	F	BG 5	14/12/2008	27/12/2008	13	27/12/2008
BG5-12/08(38)	81	F	BG 5	14/12/2008	27/12/2008	13	25/12/2008
BG5-12/08(37)	80	F	BG 5	02/12/2008	27/12/2008	25	24/12/2008
BG5-12/08(40)	89	F	BG 5	06/12/2008	27/12/2008	21	No vomiting
RH9X-12/08(41)	82	M	RH 9X	22/12/2008	29/12/2008	7	27/12/2008
RH9X-12/08(42)	83	M	RH 9X	22/12/2008	29/12/2008	7	25/12/2008
RH9X-10/08(43)	80	F	RH 9X	05/10/2008	29/12/2008	85	No vomiting
RH8A-12/08(34)	78	M	RH 8A	26/11/2008	30/12/2008	34	27/12/2008
RH3X-12/08(46)	89	F	RH 3X	17/12/2008	30/12/2008	13	24/12/2008
RH8A-12/08(45)	24	M	RH 8A	25/11/2008	30/12/2008	35	No vomiting
BG7-12/08(48)	72	M	BG 7	07/12/2008	31/12/2008	24	24/12/2008
BG5-11/08(47)	88	F	BG 5	26/11/2008	31/12/2008	35	No vomiting

Study ID (Number)	Age	Sex	Ward	Date Of admission	Date of oral sampling/data collection	Duration of admission at oral sampling: Days	Date of Onset: Vomiting
BG7-12/08(49)	92	F	BG 7	16/12/2008	31/12/2008	15	No vomiting
AP43-01/09(52)	64	F	AP 43	05/01/2009	09/01/2009	4	09/01/2009
AP43-01/09(50)	91	F	AP 43	13/12/2008	09/01/2009	27	08/01/2009
AP43-01/09(51)	87	F	AP 43	05/01/2009	09/01/2009	4	08/01/2009
AP43-01/09(53)	76	F	AP 43	06/01/2009	09/01/2009	3	No vomiting
BG5-01/09(55)	66	M	BG 5	09/12/2008	10/01/2009	32	10/01/2009
BG5-01/09(54)	86	F	BG 5	05/01/2009	10/01/2009	5	No vomiting
AP24-01/09(60)	94	F	AP 24	30/12/2008	13/01/2009	14	12/01/2009
AP23-01/09(65)	89	F	AP 23	08/01/2009	13/01/2009	5	13/01/2009
AP24-01/09(57)	91	F	AP 24	06/01/2009	13/01/2009	7	11/01/2009
AP24-01/09(61)	85	F	AP 24	31/12/2008	13/01/2009	13	10/01/2009
AP24-01/09(58)	85	F	AP 24	07/01/2009	13/01/2009	6	10/01/2009
AP24-01/09(63)	83	F	AP 24	26/12/2008	13/01/2009	18	No vomiting
AP24-01/09(56)	88	F	AP 24	29/12/2008	13/01/2009	15	No vomiting
AP25-01/09(68)	64	M	AP 25	27/11/2008	14/01/2009	48	14/01/2009
AP32-01/09(69)	71	F	AP 32	10/01/2009	15/01/2009	5	13/01/2009
AP32-01/09(70)	75	F	AP 32	08/01/2009	15/01/2009	7	No vomiting
AP27-01/09(78)	83	M	AP 27	15/12/2008	16/01/2009	32	15/01/2009
AP43-01/09(72)	86	M	AP 43	14/01/2009	16/01/2009	2	16/01/2009
APAMAU-01/09(77)	80	M	AP AMAU	12/01/2009	16/01/2009	4	11/01/2009
AP43-01/09(74)	76	M	AP 43	09/01/2009	16/01/2009	7	15/01/2009
AP27-01/09(81)	78	M	AP 27	10/01/2009	16/01/2009	6	14/01/2009
AP27-01/09(79)	84	F	AP 27	17/12/2008	16/01/2009	30	14/01/2009
AP23-01/09(80)	85	F	AP 23	18/12/2008	16/01/2009	29	14/01/2009
AP43-01/09(75)	89	F	AP 43	09/01/2009	16/01/2009	7	14/01/2009
APAMAU-01/09(76)	93	F	AP AMAU	07/01/2009	16/01/2009	9	14/01/2009
BG5-01/09(71)	75	F	BG 5	16/12/2008	16/01/2009	31	12/01/2009
AP27-01/09(82)	66	M	AP 27	24/12/2008	16/01/2009	23	No vomiting
AP43-01/09(73)	87	F	AP 43	02/01/2009	16/01/2009	14	No vomiting
AP38-01/09(86)	77	M	AP 38	02/01/2009	18/01/2009	16	18/01/2009
AP38-01/09(87)	64	M	AP 38	08/01/2009	18/01/2009	10	17/01/2009
AP38-01/09(88)	82	M	AP 38	09/01/2009	18/01/2009	9	No vomiting
AP37-01/09(85)	61	M	AP 37	09/01/2009	18/01/2009	9	No vomiting
AP27-01/09(84)	89	F	AP 27	05/12/2008	18/01/2009	44	No vomiting
BG11-01/09(70)	93	M	BG 11	10/11/2008	19/01/2009	70	19/01/2009
BG11-01/09(96)	91	F	BG 11	03/01/2009	19/01/2009	16	17/01/2009
BG11-01/09(94)	94	F	BG 11	03/01/2009	19/01/2009	16	17/01/2009
AP37-01/09(90)	70	M	AP 37	13/01/2009	19/01/2009	6	No vomiting
AP27-01/09(91)	88	F	AP 27	13/01/2009	19/01/2009	6	No vomiting
AP26-01/09(101)	91	F	AP 26	30/12/2008	20/01/2009	21	20/01/2009

Study ID (Number)	**Age**	**Sex**	**Ward**	**Date Of admission**	**Date of oral sampling/data collection**	**Duration of admission at oral sampling: Days**	**Date of Onset: Vomiting**
AP27-01/09(106)	79	M	AP 27	08/01/2009	20/01/2009	12	19/01/2009
AP27-01/09(105)	95	M	AP 27	03/01/2009	20/01/2009	17	19/01/2009
AP26-01/09(102)	61	F	AP 26	16/01/2009	20/01/2009	4	20/01/2009
BG11-01/09(100)	74	F	BG 11	07/11/2008	20/01/2009	74	19/01/2009
AP37-01/09(107)	74	F	AP 37	17/01/2009	20/01/2009	3	19/01/2009
AP26-01/09(103)	53	F	AP 26	16/01/2009	20/01/2009	4	No vomiting
AP26-01/09(104)	84	M	AP 26	14/01/2009	20/01/2009	6	No vomiting
AP27-01/09(109)	77	M	AP 27	06/01/2009	21/01/2009	15	21/01/2009
AP38-01/09(110)	80	M	AP 38	16/01/2009	21/01/2009	5	21/01/2009
BG11-01/09(108)	55	M	BG 11	16/01/2009	21/01/2009	5	No vomiting
AP37-01/09(111)	55	M	AP 37	17/01/2009	22/01/2009	5	21/01/2009
RH8MCU-01/09(112)	76	M	RH 8MCU	17/12/2008	22/01/2009	36	22/01/2009
AP26-01/09(113)	66	F	AP 26	11/01/2009	23/01/2009	12	21/01/2009
RH3A-01/09(115)	88	F	RH 3A	23/01/2009	27/01/2009	4	No vomiting
RH5B-01/09(117)	70	F	RH 5B	16/09/2008	30/01/2009	136	29/01/2009
CTA-02/09(120)	57	F	CT A	28/01/2009	03/02/2009	6	02/02/2009
CTA-02/09(121)	78	M	CT A	28/01/2009	03/02/2009	6	01/02/2009
CTA-02/09(122)	56	M	CT A	29/01/2009	03/02/2009	5	No vomiting
CTA-02/09(123)	86	M	CT A	30/01/2009	03/02/2009	4	No vomiting

Study ID (Number)	No. of days since vomiting began	Date of onset: Diarrhoea	No. of days since diarrhoea began	Last Vomit: Days	Last diarrhoea: Days	Oral real time PCR result
AP37-11/08(3)	1	23/11/2008	1	0.75	0.75	NEG
AP37-11/08(4)	2	22/11/2008	2	1.00	2.00	NEG
AP37-11/08(2)	5	19/11/2008	5	4.00	0.00	NEG
WHC3-11/08(7)	3	23/11/2008	2	0.00	0.17	NEG
AP37-11/08(11)	7	18/11/2008	7	3.00	1.00	NEG
WHC3-11/08(5)	N/A	24/11/2008	1	N/A	0.00	NEG
AP37-11/08(10)	N/A	22/11/2008	3	N/A	0.00	NEG
WHC3-11/08(8)	N/A	25/11/2008	0	N/A	0.16	NEG
WHC3-11/08(6)	N/A	22/11/2008	3	N/A	0.75	NEG
AP37-11/08(9)	N/A	25/11/2008	0	N/A	0.00	NEG
WHC3-11/08(13)	3	23/11/2008	3	0.00	0.00	NEG
WHC3-11/08(14)	3	23/11/2008	3	0.13	0.00	NEG
WHC3-11/08(12)	1	26/11/2008	0	0.25	0.00	NEG
WHC3-11/08(15)	0	26/11/2008	0	0.25	0.16	NEG
RH8B-12/08(16)	23	10/11/2008	23	N/K	1.00	NEG
WHF1-12/08(23)	15	26/11/2008	15	0.33	0.20	NEG
WHB2-12/08(26)	6	05/12/2008	6	1.00	1.00	NEG
WHF1-12/08(24)	2	09/12/2008	2	1.00	1.00	POS
WHB2-11/08(27)	4	07/12/2008	4	3.00	3.00	NEG
WHF1-12/08(21)	3	08/12/2008	3	3.50	2.00	NEG
WHF1-12/08(20)	N/A	08/12/2008	3	N/A	2.00	NEG
WHF1-12/08(22)	N/A	08/12/2008	3	N/A	2.00	NEG
WHB2-12/08(28)	N/A	09/12/2008	2	N/A	0.00	POS
AP33-12/08(33)	1	17/12/2008	1	0.75	0.25	POS
AP33-12/08(32)	1	17/12/2008	1	1.17	0.50	NEG
AP33-12/08(35)	3	15/12/2008	3	2.00	0.00	NEG
AP33-12/08(34)	3	15/12/2008	3	2.00	0.00	POS
AP33-12/08(30)	4	14/12/2008	4	4.00	1.50	NEG
AP33-12/08(31)	N/A	16/12/2008	2	N/A	1.00	NEG
BG5-12/08(39)	0	27/12/2008	0	0.33	0.16	NEG
BG5-12/08(38)	2	25/12/2008	2	0.33	0.07	POS
BG5-12/08(37)	3	24/12/2008	3	3.00	0.00	NEG
BG5-12/08(40)	N/A	26/12/2008	1	N/A	1.00	POS
RH9X-12/08(41)	2	27/12/2008	2	1.50	1.15	NEG
RH9X-12/08(42)	4	28/12/2008	1	2.00	0.13	NEG
RH9X-10/08(43)	N/A	27/12/2008	2	N/A	0.00	NEG
RH8A-12/08(34)	3	27/12/2008	3	3.50	2.50	NEG
RH3X-12/08(46)	6	24/12/2008	6	4.00	2.50	NEG
RH8A-12/08(45)	N/A	27/12/2008	3	N/A	1.00	NEG
BG7-12/08(48)	7	24/12/2008	7	7.00	1.00	NEG
BG5-11/08(47)	N/A	28/12/2008	3	N/A	0.25	NEG

Study ID (Number)	No. of days since vomiting began	Date of onset: Diarrhoea	No. of days since diarrhoea began	Last Vomit: Days	Last diarrhoea: Days	Oral real time PCR result
BG7-12/08(49)	N/A	29/12/2008	2	N/A	0.50	NEG
AP43-01/09(52)	0	09/01/2009	0	0.25	0.16	POS
AP43-01/09(50)	1	08/01/2009	1	0.77	0.75	POS
AP43-01/09(51)	1	No Diarrhoea	N/A	1.50	0.00	POS
AP43-01/09(53)	N/A	08/01/2009	1	N/A	0.00	NEG
BG5-01/09(55)	0	No Diarrhoea	N/A	0.00	0.00	POS
BG5-01/09(54)	N/A	08/01/2009	2	N/A	0.00	NEG
AP24-01/09(60)	1	12/01/2009	1	0.13	0.50	POS
AP23-01/09(65)	0	13/01/2009	0	0.20	0.15	POS
AP24-01/09(57)	2	11/01/2009	2	0.33	0.15	NEG
AP24-01/09(61)	3	10/01/2009	3	2.50	0.15	NEG
AP24-01/09(58)	3	10/01/2009	3	3.00	0.50	NEG
AP24-01/09(63)	N/A	10/01/2009	3	N/A	2.00	NEG
AP24-01/09(56)	N/A	11/01/2009	2	N/A	0,25	NEG
AP25-01/09(68)	0	14/01/2009	0	0.00	0.00	NEG
AP32-01/09(69)	2	13/01/2009	2	2.00	0.00	NEG
AP32-01/09(70)	N/A	14/01/2009	1	N/A	0.15	NEG
AP27-01/09(78)	1	15/01/2009	1	0.00	0.25	NEG
AP43-01/09(72)	0	16/01/2009	0	0.13	0.00	NEG
APAMAU-01/09(77)	5	11/01/2009	5	0.20	0.75	NEG
AP43-01/09(74)	1	15/01/2009	1	0.75	0.43	NEG
AP27-01/09(81)	2	14/01/2009	2	0.85	0.80	NEG
AP27-01/09(79)	2	14/01/2009	2	1.17	1.15	NEG
AP23-01/09(80)	2	14/01/2009	2	1.33	0.75	NEG
AP43-01/09(75)	2	13/01/2009	3	2.00	1.75	NEG
APAMAU-01/09(76)	2	14/01/2009	2	2.00	0,25	NEG
BG5-01/09(71)	4	12/01/2009	4	3.00	2.00	NEG
AP27-01/09(82)	N/A	16/01/2009	0	N/A	0.00	NEG
AP43-01/09(73)	N/A	14/01/2009	2	N/A	2.00	NEG
AP38-01/09(86)	0	18/01/2009	0	0.30	0.16	NEG
AP38-01/09(87)	1	17/01/2009	1	1.00	1.57	NEG
AP38-01/09(88)	N/A	14/01/2009	4	N/A	0.00	NEG
AP37-01/09(85)	N/A	17/01/2009	1	N/A	0.13	NEG
AP27-01/09(84)	N/A	17/01/2009	1	N/A	1.00	NEG
BG11-01/09(70)	0	19/01/2009	0	0.20	0.00	POS
BG11-01/09(96)	2	17/01/2009	2	2.00	0.60	NEG
BG11-01/09(94)	2	17/01/2009	2	2.00	0.60	POS
AP37-01/09(90)	N/A	19/01/2009	0	N/A	0.10	NEG
AP27-01/09(91)	N/A	19/01/2009	0	N/A	0.13	NEG
AP26-01/09(101)	0	No Diarrhoea	N/A	0.00	0.00	NEG

Study ID (Number)	No. of days since vomiting began	Date of onset: Diarrhoea	No. of days since diarrhoea began	Last Vomit: Days	Last diarrhoea: Days	Oral real time PCR result
AP27-01/09(106)	1	19/01/2009	1	0.00	0.00	POS
AP27-01/09(105)	1	19/01/2009	1	0.00	0.00	POS
AP26-01/09(102)	0	No Diarrhoea	N/A	0.00	0.00	POS
BG11-01/09(100)	1	19/01/2009	1	0.25	0.00	POS
AP37-01/09(107)	1	19/01/2009	1	0.25	0.33	POS
AP26-01/09(103)	N/A	20/01/2009	0	N/A	0.13	NEG
AP26-01/09(104)	N/A	20/01/2009	0	N/A	0.00	POS
AP27-01/09(109)	0	21/01/2009	0	0.00	0.00	POS
AP38-01/09(110)	0	21/01/2009	0	0.40	0.50	POS
BG11-01/09(108)	N/A	19/01/2009	2	N/A	0.75	NEG
AP37-01/09(111)	1	21/01/2009	1	0.13	0.00	NEG
RH8MCU-01/09(112)	0	22/01/2009	0	0.18	0.33	NEG
AP26-01/09(113)	2	21/01/2009	2	0.85	0.15	NEG
RH3A-01/09(115)	N/A	27/01/2009	0	N/A	0.13	NEG
RH5B-01/09(117)	1	29/01/2009	1	0.50	0.57	NEG
CTA-02/09(120)	1	02/02/2009	1	0.85	0.80	NEG
CTA-02/09(121)	2	30/01/2009	4	3.00	0.13	NEG
CTA-02/09(122)	N/A	31/01/2009	3	N/A	0.20	NEG
CTA-02/09(123)	N/A	31/01/2009	3	N/A	1.00	NEG

Study ID (Number)	**Oral qualitative PCR result**	**Faeces oral PCR result**	**Norovirus sequence obtained from faecal sample**	**Patient in laboratory confirmed outbreak**
AP37-11/08(3)	N/A	POS	YES	Y
AP37-11/08(4)	N/A	POS	YES	Y
AP37-11/08(2)	N/A	NEG	N/A	Y
WHC3-11/08(7)	N/A	POS	NO	Y
AP37-11/08(11)	N/A	POS	YES	Y
WHC3-11/08(5)	N/A	NO SAMPLE	N/A	Y
AP37-11/08(10)	N/A	POS	YES	Y
WHC3-11/08(8)	N/A	NEG	N/A	Y
WHC3-11/08(6)	N/A	POS	YES	Y
AP37-11/08(9)	N/A	POS	YES	Y
WHC3-11/08(13)	N/A	NO SAMPLE	N/A	Y
WHC3-11/08(14)	N/A	NO SAMPLE	N/A	Y
WHC3-11/08(12)	N/A	NO SAMPLE	N/A	Y
WHC3-11/08(15)	N/A	POS	NO	Y
RH8B-12/08(16)	N/A	POS	YES	Y
WHF1-12/08(23)	N/A	POS	YES	Y
WHB2-12/08(26)	N/A	NO SAMPLE	N/A	Y
WHF1-12/08(24)	NEG	NO SAMPLE	N/A	Y
WHB2-11/08(27)	N/A	NO SAMPLE	N/A	Y
WHF1-12/08(21)	N/A	NO SAMPLE	N/A	Y
WHF1-12/08(20)	N/A	NO SAMPLE	N/A	Y
WHF1-12/08(22)	N/A	POS	YES	Y
WHB2-12/08(28)	NEG	POS	YES	Y
AP33-12/08(33)	NEG	POS	YES	Y
AP33-12/08(32)	N/A	POS	NO	Y
AP33-12/08(35)	N/A	NEG	N/A	Y
AP33-12/08(34)	NEG	NO SAMPLE	N/A	Y
AP33-12/08(30)	N/A	POS	YES	Y
AP33-12/08(31)	N/A	POS	YES	Y
BG5-12/08(39)	N/A	NO SAMPLE	N/A	Y
BG5-12/08(38)	POS	POS	YES	Y
BG5-12/08(37)	N/A	NO SAMPLE	N/A	Y
BG5-12/08(40)	NEG	NO SAMPLE	N/A	Y
RH9X-12/08(41)	N/A	POS	YES	Y
RH9X-12/08(42)	N/A	POS	YES	Y
RH9X-10/08(43)	N/A	NO SAMPLE	N/A	Y
RH8A-12/08(34)	N/A	POS	NO	Y
RH3X-12/08(46)	N/A	POS	YES	Y
RH8A-12/08(45)	N/A	NO SAMPLE	N/A	N
BG7-12/08(48)	N/A	POS	NO	Y
BG5-11/08(47)	N/A	POS	YES	Y

Study ID (Number)	Oral qualitative PCR result	Faeces oral PCR result	Norovirus sequence obtained from faecal sample	Patient in laboratory confirmed outbreak
BG7-12/08(49)	N/A	POS	NO	Y
AP43-01/09(52)	POS	POS	YES	Y
AP43-01/09(50)	NEG	POS	YES	Y
AP43-01/09(51)	NEG	NO SAMPLE	N/A	Y
AP43-01/09(53)	N/A	POS	YES	Y
BG5-01/09(55)	POS	POS	YES	Y
BG5-01/09(54)	N/A	POS	YES	Y
AP24-01/09(60)	POS	NO SAMPLE	N/A	Y
AP23-01/09(65)	POS	POS	YES	Y
AP24-01/09(57)	N/A	POS	YES	Y
AP24-01/09(61)	N/A	NO SAMPLE	N/A	Y
AP24-01/09(58)	N/A	NO SAMPLE	N/A	Y
AP24-01/09(63)	N/A	POS	YES	Y
AP24-01/09(56)	N/A	POS	YES	Y
AP25-01/09(68)	N/A	NO SAMPLE	N/A	N
AP32-01/09(69)	N/A	NO SAMPLE	N/A	N
AP32-01/09(70)	N/A	NEG	N/A	N
AP27-01/09(78)	N/A	NO SAMPLE	N/A	Y
AP43-01/09(72)	N/A	NO SAMPLE	N/A	Y
APAMAU-01/09(77)	N/A	NEG	N/A	N
AP43-01/09(74)	N/A	POS	YES	Y
AP27-01/09(81)	N/A	POS	NOT TESTED	Y
AP27-01/09(79)	N/A	POS	YES	Y
AP23-01/09(80)	N/A	NO SAMPLE	N/A	Y
AP43-01/09(75)	N/A	POS	YES	Y
APAMAU-01/09(76)	N/A	POS	YES	Y
BG5-01/09(71)	N/A	POS	YES	Y
AP27-01/09(82)	N/A	POS	NO	Y
AP43-01/09(73)	N/A	NO SAMPLE	N/A	Y
AP38-01/09(86)	N/A	POS	NO	Y
AP38-01/09(87)	N/A	POS	YES	Y
AP38-01/09(88)	N/A	POS	YES	Y
AP37-01/09(85)	N/A	POS	YES	Y
AP27-01/09(84)	N/A	POS	NOT TESTED	Y
BG11-01/09(70)	NEG	POS	NO	Y
BG11-01/09(96)	N/A	POS	YES	Y
BG11-01/09(94)	POS	POS	YES	Y
AP37-01/09(90)	N/A	NO SAMPLE	N/A	Y
AP27-01/09(91)	N/A	NO SAMPLE	N/A	Y
AP26-01/09(101)	N/A	NO SAMPLE	N/A	Y

Study ID (Number)	Oral qualitative PCR result	Faeces oral PCR result	Norovirus sequence obtained from faecal sample	Patient in laboratory confirmed outbreak
AP27-01/09(106)	POS	NO SAMPLE	N/A	Y
AP27-01/09(105)	NEG	POS	YES	Y
AP26-01/09(102)	POS	NO SAMPLE	N/A	Y
BG11-01/09(100)	NEG	POS	NO	Y
AP37-01/09(107)	NEG	NO SAMPLE	N/A	Y
AP26-01/09(103)	N/A	POS	YES	Y
AP26-01/09(104)	POS	POS	NO	Y
AP27-01/09(109)	NEG	POS	YES	Y
AP38-01/09(110)	NEG	POS	YES	Y
BG11-01/09(108)	N/A	POS	NO	Y
AP37-01/09(111)	N/A	NO SAMPLE	N/A	Y
RH8MCU-01/09(112)	N/A	NEG	N/A	N
AP26-01/09(113)	N/A	NO SAMPLE	N/A	Y
RH3A-01/09(115)	N/A	NO SAMPLE	N/A	N
RH5B-01/09(117)	N/A	NEG	N/A	N
CTA-02/09(120)	N/A	POS	NP	Y
CTA-02/09(121)	N/A	POS	YES	Y
CTA-02/09(122)	N/A	POS	YES	Y
CTA-02/09(123)	N/A	POS	NO	Y

Norovirus sequence data from the study into the oral diagnosis of norovirus, UK.

Samples labelled with study number: F=faecal sample, SL = oral (saliva) sample.

>3F

ATGAAGATGGCGTCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
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>4F

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>6F

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>9F

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>10F

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>11F

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>16F

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>22F

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>23F

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>28F

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>30F

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>31F

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>33F

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>38F

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>38SL

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>41F

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>42F

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>46F

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>47F

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>50F

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>52F

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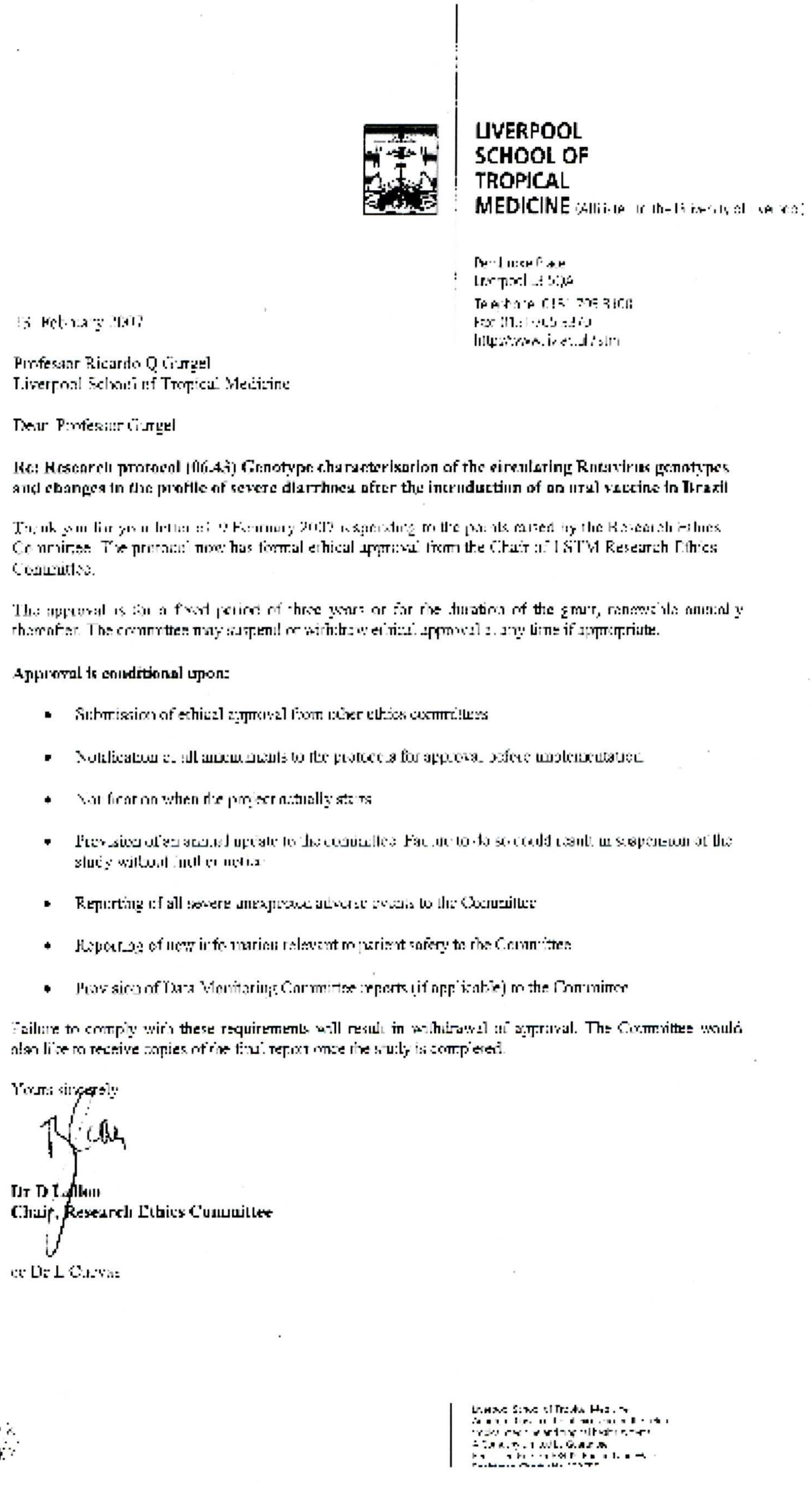
Research ethics approval: Brazilian study into paediatric gastroenteritis.



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15 February 2007

Professor Ricardo Q Gurgel
Liverpool School of Tropical Medicine

Dear Professor Gurgel

Re: Research protocol (06.43) Genotype characterisation of the circulating Rotavirus genotypes and changes in the profile of severe diarrhoea after the introduction of an oral vaccine in Brazil

Thank you for your letter of 9 February 2007 responding to the points raised by the Research Ethics Committee. The protocol now has formal ethical approval from the Chair of LSTM Research Ethics Committee.

The approval is for a fixed period of three years or for the duration of the grant, renewable annually thereafter. The committee may suspend or withdraw ethical approval at any time if appropriate.

Approval is conditional upon:

- Submission of ethical approval from other ethics committees
- Notification of all amendments to the protocol for approval before implementation
- Notification when the project actually starts
- Provision of an annual update to the committee. Failure to do so could result in suspension of the study without further notice
- Reporting of all severe unexpected adverse events to the Committee
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements will result in withdrawal of approval. The Committee would also like to receive copies of the final report once the study is completed.

Yours sincerely

Dr D Lalloo
Chair, Research Ethics Committee

cc Dr L Cuevas

ACCE evaluation for immunological based norovirus testing.

Disorder/Assay Background

Is an infectious organism or a specific condition caused by an unknown infectious organism, being investigated?

An infectious organism is being studied.

What is the infectious organism or condition being studied?

Norovirus

What is the history, clinical findings and prognoses of this disease?

The infection causes acute gastroenteritis characterised by vomiting and diarrhoea. The prognosis is different in different cohorts. In healthy adults and children it probably has negligible mortality. In children with poor overall health it is not known how much norovirus contributes to morbidity, both from acute infection and its contribution to the ongoing heavy burden of infections faced by this cohort.

What is the clinical and molecular epidemiology of this disease? Are there public health/political implications?

Clinical epidemiology: Norovirus causes infections in all age groups in all geographical locations. It is particularly recognised as affecting people in institutions e.g. schools and hospitals. In these setting norovirus causes outbreaks affecting large numbers of people with impairment of that institutions ability to function. This has significant cost implications. Approximately 20% of the population are resistant to infection with norovirus as they do not have receptors in the gastrointestinal tract for the virus to bind to (non-secretors).

Molecular epidemiology: Norovirus is classified into genogroups of which genogroup I and II are the main causes of human infection. The genogroups are separated in genotypes, of which GII.4 is the cause of the majority of infections worldwide.

Public health implications: Norovirus causes outbreaks of infections in institutions with significant cost implications for these institutions which affects spending in other areas. Otherwise, in developed countries there is limited morbidity and mortality caused by norovirus. The effect on public health in developing countries is not known and may be greater given the relatively poor health in many countries.

Political Implications:

Norovirus is a political issue given the adverse effect it has on public institutions.

What therapy is available? Is empirical therapy available? What are the side effects of therapy?

No specific therapy is available. Supportive therapy can be given as for any gastroenteritis.

What is the clinical setting in which the test is to be performed?

ELISA and ICG norovirus tests: Clinical laboratories serving paediatric populations with significant health resources to cover the cost of this testing.

What microbiological assays / non microbiological investigations are associated with this disorder? Which are you evaluating?

Electron Microscopy. This has 30% sensitivity when compared to PCR and is not planned to be evaluated.

ELISA. A new ELISA assay is available for norovirus testing, the RIDASCREEN norovirus test. Evaluation of this test is planned.

ICG testing. A new ICG assay is available for PCR testing, the RIDAQUICK norovirus test. Evaluation of this test is planned.

PCR. This method is established as the gold standard test and is planned as the reference method in this study.

How long does the assay take? How does this relate to the time course of the infection and other diagnostic assays?

ELISA. This takes a minimum of 3 hours and is designed for the testing of multiple samples.

ICG. This takes a minimum of 20 minutes and is designed for the testing of individual samples

PCR. This takes a minimum of 4 hours and is designed for the testing of multiple samples.

Norovirus infection has an incubation period of 24 hours and duration of infection of approximately 48 hours. The tests may therefore relate to the time course of the infection in a number of different ways depending on the clinical situation and laboratory situation. For example, in a hospital setting it may be important to get a rapid diagnosis to improve infection control.

What time period in relation to an illness is the test relevant to?

ELISA/ICG. The test is relevant from the onset of symptoms up to7 days following infection, but it is recommended it be used in the first 72 hours of the illness.

PCR. The test is relevant form the onset of symptoms and will remain positive for on average 1 month.

Are the assays used for screening/diagnostic purposes? Are the assays useful to rule in/rule out a condition?

The tests may be used for diagnostic or screening purposes.

Is the assay completed within the same assay as others? Is the assay completed at the same time as other assays (parallel) or on the basis of other results (series)? Does the result lead to further assays/investigations being completed?

Norovirus testing is usually completed independently of other tests, although it may be common for other enteropathogens to be tested for at the same time. The norovirus tests themselves are usually carried out in isolation although one testing strategy may be to complete immunological testing prior to PCR testing.

What is the laboratory setting in which the assay is to be performed?

Immunological tests will be completed at district general hospitals and regional hospitals where PCR testing will be more commonly completed in regional hospitals.

Are there developments visible in the future (diagnostic, therapeutic or preventative that will affect the durability of the implications of the ACCE assessment? Is an ACCE analysis for this test a priority?

Nothing obvious.

Using the information obtained by answering the above define the clinical care pathway this assay fits into.

Given the absence of treatment for norovirus and the limited morbidity it causes norovirus testing will be limited to countries with significant health care resources, and often just to the investigation of outbreaks of infection.

Analytic Validity

Is the assay qualitative or quantitative?

Qualitative

How often is the test positive/negative when the infectious organism is present (analytic sensitivity) / absent (analytic specificity)?

In comparison to PCR the sensitivities identified in this study are.

Test	Sensitivity	Specificity (%)	PPV	NPV
RIDASCREEN hospital and	64	100	100	95
RIDASCREEN hospital	70	100	100	94
RIDASCREEN community	50	100	100	95
RIDAQUICK hospital and	72	100	100	96
RIDAQUICK hospital	80	100	100	96
RIDAQUICK community	47	100	100	95
IDEIA hospital and community	49	100	100	93
IDEIA hospital	62	100	100	93
IDEIA community	20	100	100	93

Is an internal quality control program defined and externally monitored?

This will be location specific

Have repeated measurements been made on specimens (e.g. positive/negative controls)?

Reproducibility has been assessed and found to be satisfactory for results outside the equivalent range. Within this range significant (50%) variation in results is seen.

What is the within- and between-laboratory precision?

Not known.

How similar are results obtained in multiple laboratories using the same, or different technology?

Not known.

If appropriate, how is confirmatory testing performed to resolve false positive results, how long does this take?

The tests are highly specific so confirming positive results is not needed.

What range/quality of patient specimens has been tested?

Samples collected in the community and hospital were tested form children with modified Vesikari severity scores of 8 and 10 respectively.

How often does the test fail to give a useable result?

The RIDASCREEN an IDEIA ELISA gave an equivocal result in 5 and 3 of 212 samples tested respectively.

Clinical Validity

How often is the test positive/negative when the infectious disease is present (clinical sensitivity) / absent (clinical specificity)?

This is difficult to answer as the gold standard detects shedding (identifies the infectious disease as present when it is absent). If that the rate if clinical positive infections is assumed to be 16.8% (hospital rate) minus the rate of asymptomatic shedding (8%) the level of clinical positives is 8.8% The ELISA tests detect approximately 70% of hospital PCR positives, =11.8%. On these assumptions the ELISA tests, given false positives are not seen, are likely to detect all clinical positive infections and some asymptomatic shedding (2.0% (11.8-8.8).

Clinical specificity: the rate of ELISA/ICG positives in those without disease may be approximately 2%, see section 3.2.2.2 for calculations.

What is the prevalence of the disorder in this setting? What are the positive and negative predictive values?

In the hospital setting the PPV of the RIDASCREEN is approximately 75%, see discussion. The negative predictive value will be high as the prevalence is low.

Are there methods to resolve clinical false positive results in a timely manner?

The clinical history and results of other tests offer the only current means of resolving clinical false positives.

Has the test been adequately validated on all populations to which it may be offered?

This evaluation assesses the RIDASCREEN/RIDAQUICK norovirus tests in paediatric populations with diarrhoea in the community and presenting to hospital. They have not been evaluated outside these cohorts.

What are the relationship between presence of organism and disease (e.g. colonisers/obligate pathogens)?

Following infection with norovirus there is prolonged shedding of norovirus in children, an average as detected by PCR of approximately 35 days (Kirkwood and Streitberg 2008) in one study and 19 days in another (Murata et al. 2007), up to 100 days. Norovirus shedding as detectable by ELISA based assays has only been assessed in adults, in one human challenge study antigen was detectable for a median of 7 days (28 by PCR) (Atmar et al. 2008) .

Infection may though be symptomatic or asymptomatic. In adult challenge studies only half of infection (detected by PCR) is symptomatic (Lindesmith et al. 2003).

What affects the relationship between organism and disease (e.g. immunosuppression, prosthetic material)?

Prior infection is believed to offer protection against disease (Lindesmith et al. 2005) for a period of 6-12 months. Disease duration is prolonged in hospitalised patients. Morbidity and mortality are influenced by co-morbidities e.g. those with severe cardiac impairment may not tolerate dehydration well.

Clinical Utility

What is the impact of a positive (or negative) test on patient care?

There is no specific treatment for norovirus so there will only be indirect benefits to a patient in having this diagnosis made. These indirect benefits may be in making a diagnosis, so preventing the need for further investigations and alternative therapies e.g. antimicrobial therapy.

What is the impact on the prevention of spread of infection?

Norovirus may spread in institutions which children attend causing outbreaks. Making a diagnosis of the aetiological agent of an outbreak may ensure the optimal control strategies are implemented.

Is there an effective treatment or other measurable benefit? Is there general access to that treatment or benefit?

No specific therapy.

Is the test being offered to a socially vulnerable population?

Location specific

What quality assurance measures are in place?

Location specific

What health risks can be identified for the intervention?

A false positive result may lead to another infection/condition not being treated.

What are the economic costs associated with testing and what are the economic benefits resulting from testing?

Location specific

What are the results of pilot trials?

None

What facilities/personnel are available or easily put in place?

Location specific

Are there informed consent requirements?

No

What methods exist for long term monitoring?

N/A

What guidelines have been developed for evaluating clinical care pathway performance?

None

ELSI

What is known about transmission of infection, stigmatization, discrimination, confidentiality and personal/family social issues?

Norovirus testing will not lead to any of these problems.

Are there legal issues regarding consent, ownership of data and/or samples, patents, licensing, proprietary testing, obligation to disclose, or reporting requirements?

No

Other

The RIDASCREEN ELISA and RIDAQUICK ICG use monoclonal antibodies as opposed to polyclonal antibodies. This is preferable given the animal involvement in producing polyclonal antibodies.

Summary of the main features of Brazilian study site.

Table A2: Database of results for the evaluation of the IDEIA, RIDASCREEN and RIDAQUICK norovirus tests on norovirus positive samples (by PCR).

Participant Number	Date	Hospital/Community	Genogroup	Genotype	IDEIA	RIDASCREEN	RIDAQUICK
1	10/06	H	2	**G2.4**	N	P	P
2	11/06	H	2	**G2.4**	P	P	P
3	11/06	H	2	**G2.4**	P	P	P
4	11/06	H	2	**G2.4**	N	P	P
5	12/06	H	2	.	P	P	P
6	12/06	H	2	**G2.4**	P	P	P
7	12/06	H	2	**G2.4**	P	P	P
8	12/06	H	2	**G2.2**	N	P	P
9	12/06	H	2	**G2.2**	N	N	N
10	12/06	H	2	**G2.2**	P	P	P
11	12/06	H	2	**G2.4**	P	P	P
12	03/07	H	1	**G1.12**	N	N	N
13	03/07	H	1	**G1.3**	N	N	N
14	03/07	H	1	**G1.7**	N	N	N
15	03/07	H	2	.	P	P	P
16	03/07	H	2	**G2.4**	P	P	P
17	04/07	H	2	**G2.4**	P	P	P
18	04/07	H	2	**G2.4**	P	N	P
19	04/07	H	2	**G2.4**	P	P	P
20	04/07	H	2	**G2.4**	N	N	P
21	04/07	H	2	**G2.4**	P	P	P
22	04/07	H	2	**G2.4**	P	P	P
23	05/07	H	2	**G2.4**	P	P	P
24	05/07	H	2	**G2.4**	N	P	P
25	05/07	H	2	**G2.4**	P	P	P
26	05/07	H	2	**G2.4**	N	P	P
27	05/07	H	2	**G2.4**	P	P	P
28	06/07	H	2	**G2.4**	P	P	P
29	06/07	H	2	**G2.4**	P	P	P
30	06/07	H	2	**G2.4**	P	P	P
31	06/07	H	2	**G2.13**	N	N	P
32	06/07	H	2	.	N	P	P
33	06/07	H	2	**G2.4**	P	P	P
34	06/07	H	1	**G1.14**	N	N	N
35	06/07	H	2	**G2.4**	P	P	P
36	06/07	H	2	**G2.4**	N	N	P
37	06/07	H	2	**G2.4**	P	P	P
38	07/07	H	2	**G2.4**	P	P	P
39	07/07	H	2	**G2.4**	P	P	P
40	07/07	H	2	.	N	N	N
41	07/07	H	2	**G2.4**	P	P	P
42	07/07	H	2	**G2.13**	P	P	P
43	07/07	H	2	**G2.4**	P	P	P
44	07/07	H	2	**G2.4**	P	P	P
45	09/07	H	2	.	P	P	P
46	09/07	H	2	**G2.4**	P	P	P
47	09/07	H	2	**G2.4**	P	P	N
48	09/07	H	2	**G2.4**	P	P	P
49	09/07	H	2	**G2.4**	P	P	P
50	10/07	H	2	**G2.4**	P	N	P
51	10/07	H	2	**G2.4**	N	N	P
52	10/07	H	1	**G1.14**	N	P	N
53	10/07	H	2	**G2.4**	P	P	P

54	10/07	H	2	**G2.4**	N	N	P
55	10/07	H	1	**G1.14**	N	N	N
56	10/07	H	2	**G2.4**	P	P	P
57	10/07	H	2	**G2.4**	P	P	P
58	10/07	H	2	**G2.4**	P	P	P
59	10/07	H	2	**G2.4**	P	P	P
60	10/07	H	2	**G2.4**	N	N	P
61	10/07	H	2	**G2.4**	P	P	P
62	10/07	H	2	.	N	N	N
63	10/07	H	2	.	N	N	N
64	11/07	H	2	.	N	N	N
65	12/07	H	2	**G2.4**	N	N	N
66	12/07	H	2	**G2.4**	N	N	N
67	nk	C	2	**G2.2**	N	N	N
68	04/07	C	1	**G1.3**	N	N	N
69	08/07	C	2	**G2.13**	N	P	P
70	05/07	C	2	**G2.13**	N	P	N
71	05/07	C	2	**G2.13**	N	P	P
72	05/07	C	2	**G2.13**	N	P	N
73	06/2007	C	2	**G2.4**	N	P	P
74	12/07	C	2	**G2.4**	N	N	N
75	01/07	C	1/2	**G1.14**	N	P	P
76	06/07	C	2	.	N	N	N
77	06/07	C	2	.	N	N	N
78	06/07	C	2	.	N	N	N
79	04/07	C	2	.	N	N	N
80	08/07	C	2	**G2.13**	N	N	P
81	08/07	C	2	.	N	N	P
82	08/07	C	2	**G2.6**	N	P	P
83	09/07	C	2	**G2.4**	N	N	N
84	06/07	C	2	**G2.4**	P	P	P
85	06/07	C	1	.	N	N	N
86	08/07	C	2	**G2.4**	P	P	P
87	11/07	C	2	**G2.4**	P	P	P
88	09/07	C	2	.	P	P	P
89	10/07	C	2	**G2.4**	N	N	N
90	09/07	C	2	**G2.4**	N	N	N
91	11/07	C	2	**G2.4**	N	P	P
92	11/07	C	1/2	**G1.14**	N	P	N
93	12/11	C	2	**G2.4**	P	P	P
94	10/07	C	2	**G2.4**	P	P	P
95	01/08	C	2	.	N	N	N
96	10/07	C	2	.	N	N	N

Norovirus sequence data from the study on infectious gastroenteritis in Brazil.

>H10/06(1),H03/07(16),H11/06(3),H12/06(7), C09/07(90), H11/06(4), H11/06(2), H04/07(19), C06/07(73), C06/07(84), H05/07(23), H05/07(24), H0507(25), H12/06(11), H12/06(11), H05/07(25)

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>H06/07(33), H06/07(36), H06/07(37), H07/07(38) H07/07(39)

ATGAAGATGGCGTCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAGCCTGTTGTCGGTGCCGCTATTGCGGCGCCTGTAGCGGGCCAAACAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTAGGC
CCTGATCTGAATCCCTACCTATCTCATTTGGCCAGA

>H06/07(34), H10/07(55)

ATGATGATGGCGTCTAAGGACGCCCCAACAAACATGGATGGCACCAGTGGTGCCGGTCAGCTGGTTCCAGAGGCCAGTACA
GCTGAACCTATATCAATGGAACCTGTGGCTGGTGCTGCTACAGCAGCAGCAACCGCAGGTCAAGTTAATATGATTGACCCCT
GGATTATGAGTAATTATGTTCAAGCTCCCCAAGGAGAGTTCACAATTTCCCCTAACAATACCCCCGGAGACATATTGTTTGAT
TTACAATTAGGCCCCCATTTAAATCCATTCCTATCACACCTAGCCCAAATGTA

>H07/07(41)

ATGAAGATGGCGTCGAGTGACGCCAACTCCATTCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTAT
GGCTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAAT
AATTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGG
GCCCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGAATGTA

>H07/07(43)

ATGAAGATGGCGTCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAGCCCGTTGTCGGTGCCGCTATTGCGGCGCCTGTAGCGGGCCAAACAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTAGGC
CCTGATCTGAATCCCTACCTATCTCATTTGGCCAGAATGTA

>H07/07(44), H10/07(51), H10/07(54), H10/07(57), H12/07(66), H10/07(56), C10/07(94)2V2

ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGGC
CCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGA

>H09/07(46)

ATGAAGATGGCGCCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAGCCCGTTGTCGGTGCCGCTATTGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGAAACGCTCCAGGTGAGATACTATGGAGCGCGCCCTTAGGC
CCTGATCTGAATCCCTACCTATCTCATTTGGCCAGAATGTA

>H09/07(48), H09/07(49), H10/07(53), H10/07(58), H09/07(47)

ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTGTCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGGC
CCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGA

>H10/07(50)

ATGAAGATGGCGTCGAGTGACGCCAACtCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATG
GCTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATA
ATTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTGTCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGG
CCCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGAATGTA

>H10/07(52)

ATGATGGCGTCTAAGGACGCCCCAACAAACATGGATGGCACCAGTGGTGCCGGTCAGCTGGTTCCAGAGGCCAGTACAGCT
GAACCTATATCAATGGAACCTGTGGCTGGTGCTGCTACAGCAGCAGCAACCGCAGGTCAAGTTAATATGATTGACCCCTGGA
TTATGAGTAATTATGTTCAAGCTCCCCAAGGAGAGTTCACAATTTCCCCTAACAATACCCCCGGAGACATATTGTTTGATTTA
CAATTAGGCCCCCATTTAAATCCATTCCTATCACACCTAGCCCAAATTGGCCAGA

>H10/07(59)

ACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGGCTTTGGAACCCGTTGTTGG
TGCCGCTATCGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCCCGGT
GGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGG

>H10/07(61)

ATGAAGATGGCGTCGAATGACGCCAACATTCTGGATGGGTCCACAGTCCAACCTCGTCCCAGCAGGTCAACAATGAGGTTAT
GGCTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCTTGGATTACAAAT
AATTTTGTACAAGCCCXCGGTGGAGAGTTTACAGTGTCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTXGG
GCCCTGATTTGAATCCCTACCTTTCCXATTTGGCCAGAATGTA

>H12/07(65)

ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATAGCTATGGAGCGCGCCCTTGGG
CCCTGATTTGAACCCCTACCTTTCCCATTTGGCCAGAATGTA

>C11/07(93)

ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCCAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTCATGGC
TTTGGAACCCGTTGTTGGTGCCGCTATTGxxCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
TTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGGC
CCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGAATGTA

>NK(67)

ATGAAGATGGCGTCGAATGACGCCGCTCCATCTACTGATGGTGCAGCCGGCCTCGTGCCAGAAAGTAATAATGAGGTCATGG
CTCTTGAACCCGTGGCTGGTGCCGCCTTGGCAGCCCCGGTCACCGGTCAAACAAATATTATAGATCCTTGGATTAGAGCAAA
TTTTGTCCAGGCCCCCAATGGTGAATTTACAGTCTCTCCCCGTAATGCCCCTGGTGAAGTGCTACTGAATCTAGAGTTGGGTC
CAGAATTAAATCCTTATCTGGCACATTTAGCAAGACCTAT

>C08/07(69), C05/07(72), C05/07(70), C08/07(80),C01/07(75), H07/07(42)

ATGAAGATGGCGTCGAATGACGCTACTCCATCTGATGATGGTGCAGCCGGCCTCGTACCAGAGATCAACAATGAGGTTATGG
CTCTTGAACCCGTCGCTGGGGCCTCCCTTGCAGCCCCCGTAGTCGGCCAACAGAATATAATTGATCCCTGGATTAGAAATAAT
TTTGTACAAGCCCCTGCTGGTGAATTTACAGTTTCCCCTAGAAACTCTCCTGGAGAACTTCTACTTGATTTGGAATTGGGTCCT
GAACTTAATCCCTATCTTGCACATTTGGCCAGGATGTA

>C12/07(74)

ATGAAGATGGCGTCGAATGACGCTACTCTATCTGATGATGGTGCAGCCGGCCTCGTACCAGAGATCAACAATGAGGTTATGG
CTCTTGAACCCGTCGCTGGGGCCTCCCTTGCAGCCCCCGTAGTCGGCCAACAGAATATAATTGATCCCTGGATTAGAAATAAT
TTTGTACAAGCCCCTGCTGGTGAATTTACAGTTTACCCTAGAAACTCTCCTGGAGAACTTCTACTTGATTTGGAATTGGGTCCT
GAACTTAATCCCTATCTTGCACATTTGGCCAGGATGTACAACGGGCAT

>C04/07(68)

ATGGCGTCTAAGGACGCCCCAACAAACATGGATGGCACCAGTGGTGCCGGCCAGCTGGTACCAGAGGCAAACACAGCTGAG
CCTATTTCAATGGAACCAGTAGCTGGGGCTGCGACAGCAGCCGCAACTGCTGGACAAGTAAATATGATTGACCCCTGGATAA
TGAATAATTATGTGCAAGCCCCTCAAGGAGAGTTCACCATTTCGCCCAACAACACTCCTGGCGACATTTTATTTGACCTACAA
TTGGGCCCACACCTCAACCCTTTCTTATCCCATTTA

>C11/07(87)

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ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAGCCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAA
CTTTGTACAAGCCCCTGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGGC
CCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGAATGTA

>C08/07(86)

ATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCACAGCCAACCTCGTCCCAGAGGTCAACAATGAGGTTATGG
CTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAACAA
TTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTATCCCCTAGAAACGCTCCAGGTGAAATACTATGGAGCGCGCCCYTGGGC
CCTGATTTGAATCCCTACCTTTCCCATTTGGCCAGAATGTA

>C05/07(71)

ATGAAGATGGCGTCGAATGACGCTACTCCATCTGATGATGGTGCAGCCGGCCTCGTGCCAGAGATCAACAATGAGGTTATGG
CTCTTGAACCCGTCGCTGGGGCCTCCCTTGCAGCCCCCGTAGTCGGCCAACAGAATATAATTGATCCCTGGATTAGAAATAAT
TTTGTACAAGCCCCTGCTGGTGAATTTACAGTTTCCCCTAGAAACTCTCCTGGAGAACTTCTACTTGATTTGGAATTGGGTCCT
GAACTTAATCCCTATCTTGCACATTTGGCCAGG

>C10/07(89), C09/07(83)

ATGAAGATGGCGTCGAATGACGCTAATCCGTCCAGTGACGGATCAGCCAACCTCGTCCCAGAGATCAGTAATGAGGTTATGG
CCCTTGAACCAGTAGCTGGTGCTGCTATTGCTGCCCCAGTTGCTGGACAACAAAACATAATAGATCCCTGGATTAGAAACAA
TTTTGTACAGGCCCCTGGTGGTGAATTCACTGTTTCACCAAGAAACGCCCCGGGGGAAGTACTTCTAAATTTACCCTTGAGTC
CTGACATCAATCCATATTTGGCCCATCTGTCCAGAATGTA

>C01/07(75)

ATGATGGCGTCTAAGGACGCCCCAACAAACATGGATGGCACCAGTGGTGCCGGTCAGCTGGTTCCAGAGGCCAGTACAGCT
GAACCTATATCAATGGAACCTGTGGCTGGTGCTGCTACAGCAGCAGCAACCGCAGGTCAAGTTAATATGATTGACCCCTGGA
TTATGAGTAATTATGTTCAAGCTCCTCAAGGAGAGTTCACAATTTCCCCTAACAATACCCCCGGAGACATATTGTTTGATTTA
CAATTAGGCCCCCATTTAAATCCATTCCTATCACACCTAGCCCAAAGGCCAGA

>C08/07(82)

ATGAAGATGGCGTCGAATGACGCCGCTCCATCAAATGATGGTGCTGCCAACCTCGTACCAGAGGCCAACAGTGAGGTTATGG
CACTTGAACCGGTGGTGGGAGCCTCAATTGCAGCTCCTGTTGTCGGTCAGCAAAATATAATTGACCCCTGGATTAGAGAAAA
CTTTGTCCAAGCACCACAGGGCGAGTTCACTGTTTCACCAAGGAATTCGCCCGGTGAGATGCTTCTGAATCTTGAATTGGGCC
CAGAACTTAATCCCTACTTGAGTCATTTGTCCCGC

>C11/07(92)

ATGGCGTCTAAGGACGCCCCAACAAACATGGATGGCACCAGTGGTGCCGGTCAGCTGGTTCCAGAGGCCAGTACAGCTGAA
CCTATATCAATGGAACCTGTGGCTGGTGCTGCTACAGCAGCAGCAACCGCAGGTCAAGTTAATATGATTGACCCCTGGATTA
TGAGTAATTATGTTCAAGCTCCTCAAGGAGAGTTCACAATTTCCCCTAACAATACCCCCGGAGACATATTGTTTGATTTACAA
TTAGGCCCCCATTTAAATCCATTCCTATCACACCTAGCCCAAATTTGTCCCGC

>C11/07(91)

TGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGAGTGACGCCAACTCCATCTGXXATGGGTCC
ACAGCCAACCTCGTCCCAGAGGTTCAACAATGAGGTTATGGCTTTGGAACCCGTTGTTGGTGCCGCTATTGCGGCACCTGTAG
CGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCCCGGTGGAGAGTTTACAGTGTCCCCTAG
AAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGG
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